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PRESSURE-TEMPERATURE-INHIBITOR RELATIONS IN THE LUMINESCENCE OF CHAETOPTERUS VARIOPEDATUS AND ITS LUMINESCENT SECRETION [1]

HSIEN-CHOH SIE, JOSEPH JIN CHANG,[2] AND FRANK H. JOHNSON

Biology Department, Princeton University, Princeton, N. J., The Marine Biological Laboratory, Woods Hole, Massachusetts, and Biology Department, Brown University, Providence, R. I.

EIGHTEEN FIGURES

INTRODUCTION

Investigation of the influence of hydrostatic pressure, temperature, and drugs or other chemical agents on luminescent systems has a two-fold interest: first, it provides an advantageous approach to an understanding of the biological action of these factors in general, and second, it provides a means of contributing to an understanding of the process of luminescence in particular (Harvey, '52; Johnson, Eyring and Polissar, '54; Johnson, '55, '57, '58). In only one instance, however, have quantitative interrelations in the influence of pressure, temperature and drugs been investigated on the same luminescent system in both living cells and cell-free extracts, namely, bacterial luminescence (Strehler and Johnson, '54). The present study deals with the luminescence of an organism, namely *Chaetopterus*, which lends itself to a study of the pressure-temperature relations of luminescence both in whole organ systems of the animal and in an essentially cell-free slime secreted from luminescent organs.

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[2] Present address: National Institutes of Health, Bethesda, Md.

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Various aspects of light production in *Chaetopterus* have been studied by a number of investigators, as far back as Panceri (1878) (cf. Harvey, '52; Nicol, '52b). The most recent work is that by Nicol ('52a, b, c, '54a, b), on the physiology of stimulation and luminescent response. In one type of response, a luminous slime is liberated from gland cells, chiefly in the aliform notopodia, whence it may pass out through the oral groove into the medium. A second type of response occurs in the segmented posterior notopodia, and certain other parts, whereby the secretion of luminescent products is not accompanied by the liberation of appreciable amounts of the material into the medium; apparently this slime adheres to the cell surface. For convenience of discussion, "slime" in the present paper will refer to the former of these types, i.e., free slime that has been liberated into the sea water.

In the slime, once it has been secreted, the intensity of luminescence undergoes continuous decay over a period of several minutes or longer, depending on various conditions. Thus far, attempts to restore luminescence after its decay have been either unsuccessful or have yielded results of uncertain significance. Moreover, attempts to obtain a "luciferin-luciferase" reaction in materials from the luminous organs, or in the slime itself, have been unsuccessful.

In the posterior notopodia of an intact worm, single flashes of light can be induced by relatively weak, local stimuli, whereas stronger stimuli will elicit responses from one or more adjacent notopodia (Nicol, '52b). Although successive, subthreshold stimuli can elicit a response, the response is never "all or none." In the present experiments, stimulation was confined to a single excised notopodium, as described presently. The strength and frequency of stimulation were chosen according to the purpose in view.

METHODS

Keeping the animals. The experiments reported in this paper were all done with the variety of *Chaetopterus variopedatus* that occurs in the region of the Marine Biological

Laboratory at Woods Hole, Massachusetts. The collected specimens were removed from their parchment-like tubes and were placed individually in 25 × 250 mm glass tubes, constricted to about 12 mm at one end, and closed at the other end by a cork with a 12 mm hole bored through it. The glass tubes containing the organisms were placed in a large aquarium with slowly running sea water. Kept in this manner, the worms remained active and apparently healthy for an indefinite period of time.

Collecting the slime and recording its luminescence. In our experience, the individual specimens varied greatly in the amount of luminous slime that they would liberate, as a result of mechanical agitation. In fact, although all specimens when agitated became brightly luminescent in various regions of the body, only about 6 out of 20 specimens consistently produced appreciable amounts of free slime. Any one of these 6 specimens was used as a source of luminescent secretion only once in a given day. The same individual would again freely discharge the secretion after 24 hours.

In harvesting the luminescent slime, the glass tube containing the animal was first handled gently, with little disturbance to the worm, and the major portion of the sea water was carefully drained from the tube. The tube containing the worm was then shaken vigorously in a dark room. As soon as a bright luminescence appeared in the small remaining volume of sea water, the fluid was poured quickly into a 100 ml cylinder and was diluted with sea water, previously equilibrated at a desired temperature, to about 40 ml, an amount sufficient to fill the pressure chamber (fig. 1). The equilibrated fluid was then introduced at once into the pressure chamber, which had already been equilibrated at the same temperature in a light-tight, water bath. Pressure could be quickly applied or released during the decay of luminescence, intensity of which was recorded continuously by means of an Esterline-Angus automatic recorder. To apply pressure, the needle valve M was opened and the desired pressure was quickly obtained by one or two strokes of the pump. From the moment the

slime was collected, less than a minute was required to transfer it into the chamber and begin recording the intensity of the light, under normal or increased pressure.

Notopodia. To obtain a single isolated notopodium from the posterior region of a worm, the specimen was first anesthetized by placing it in ice cold sea water for 15–20 minutes. Once anesthetized, no luminescent response was induced in the worm by mechanical agitation or by the actual excision

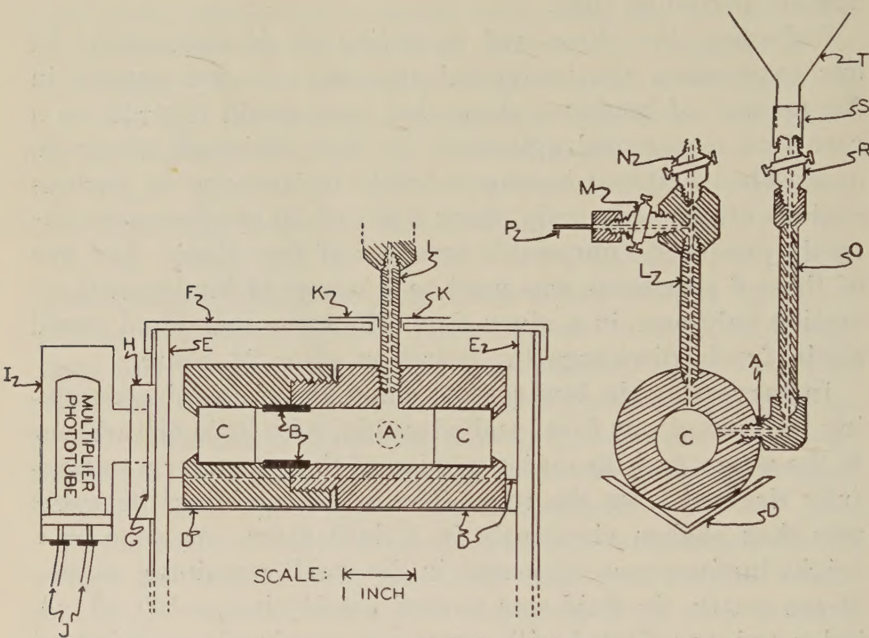

Fig. 1 Diagram of stainless steel ''bomb'' assembly for recording the intensity of luminescence in the free slime. The temperature equilibrated, diluted slime could be quickly introduced through funnel T, connected by rubber tube S to needle valve R, thence through tube O and elbow A to the chamber with Herculite plate glass windows C, finally overflowing through tube L and needle valve N. Valves N and R were then closed, and valve M opened in order to apply pressure from the hydraulic pump through line P. Before and during filling, the bomb was held by support D in a water bath with lucite walls E, shielded from light by cover F and black felt K. Light emerging through the window C reached the photomultiplier I after opening shutter G in the holder H. Leads J were connected to the power supply and amplifier and finally to the Esterline Angus recorder. Neoprene washer B sealed the chamber against leakage under pressure. The windows C were seated with high vacuum cement to seal against leakage.

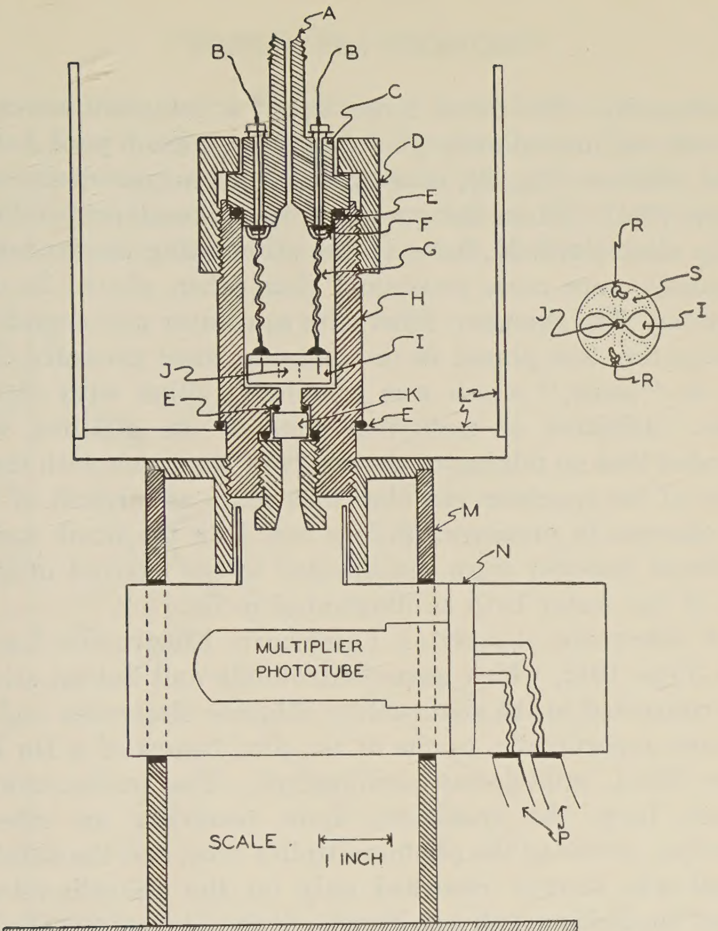

Fig. 2 Diagram of assembly for stimulating excised notopodia and recording the luminescent flashes under various hydrostatic pressures. The small insert at the right shows the lucite specimen chamber, as seen from above. The small well J, containing the specimen in sea water, was connected by a narrow opening to the electrode pools I, the whole top being covered by a lucite disc held tightly in place by screws R. To facilitate temperature equilibration, the lucite was cut away in the bottom portion leaving only thin walls to the specimen chamber and electrode pools. Silver-silver chloride electrodes passed through the covering disc, where they were insulated with sticky-wax and connected to wires G, in snug plastic tubing. These wires were soldered to screw heads F which in turn were soldered to a brass washer, then insulated with sticky-wax. A neoprene washer beneath the brass washer insulated the leads from the bomb head, and sealed against leakage under pressure where the screws passed through the bomb. A thin cylinder of Bakelite insulated the length of the screws, which communicated with the outside at C through a rubber washer held tightly in place by a brass washer under a nut. Wires B were connected to the stimulator.

For use, the main chamber of the bomb, with walls H, was filled with distilled water and equilibrated in the lucite water bath with side and base L. The removable head with specimen chamber attached by wires G was seated, and held in place by screwing on part D. Pressure was supplied by attaching the line from the hydraulic pump at A. Leakage was prevented by "O" rings E. Light from the specimen passed through plate glass window K to the photomultiplier in a light shielding housing N and a light shielding support M. Leads P from the photomultiplier were connected to the amplifier and then to the Esterline-Angus recorder, as well as to an oscillograph in some of the experiments.

of notopodia. The distal 2 mm tip of a notopodium was cut off, and was immediately placed in the specimen pool J of the lucite chamber (fig. 2), corresponding to a type designed by Chang ('54). When the specimen was placed perpendicular to the electrical field, between the stimulating electrodes, the responses were more consistent than when placed in other positions. The chamber, filled with sea water and sealed with a lucite top, was placed in the stainless steel pressure chamber, or "bomb," which was completely filled with distilled water. Addition of methylene blue to the distilled water revealed that no mixing of the water of the bomb with the sea water of the specimen chamber took place as a result of various changes in pressure. Before use, both the bomb and the specimen chamber were equilibrated to the desired temperature in the water bath as illustrated in figure 2.

An electronic stimulator (American Electronics Laboratory Type 104), which gave controllable and known stimuli, was connected to the silver-silver chloride electrodes and also in some experiments, to one of the dual beams of a Du Mont Type 322-A cathode-ray oscillograph. The luminescent response from the specimen, upon receiving an effective stimulus, activated the photomultiplier tube, and the amplified signal was usually recorded only on the Esterline-Angus. When the oscillograph was also used, simultaneous recordings were made on both instruments, the tracings on the oscillograph being photographed on a moving film. Inasmuch as the flash is relatively slow, the Esterline-Angus proved to be quite adequate for measuring the time course of light intensity, as shown by the quantitative agreement between the course of the response as recorded on the two instruments (see fig. 12*A*); the oscillograph was used mainly for determining the relation between time of stimulation and onset of response.

RESULTS

A. Slime

Decay course at different temperatures. Curves illustrating the decay in intensity of luminescence in slime are shown

in figure 3, where the logarithm of light intensity is plotted against time, for three different temperatures. The rates of decay are not linear, except for the initial portion of the decay at 10°C. The 4 curves in figure 3 pertain to slime secreted by 4 different individual worms. The rate of decay, at a given

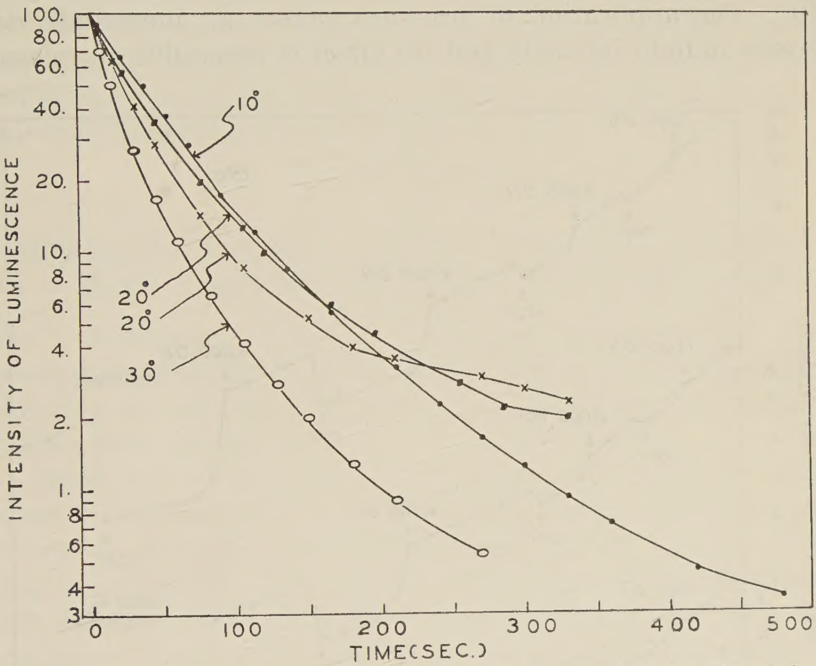

Fig. 3 Decay of luminescence in slime of *Chaetopterus* at 3 different temperatures and at normal pressure, plotted from the Esterline-Angus records. For convenience of comparison, the intensity at zero time is arbitrarily taken as 100 and subsequent intensities during the course of decay multiplied by the appropriate factor.

temperature, was found to vary somewhat among different individuals. Moreover, since the times required for collecting and transferring specimens to the bomb could not be made identical, slopes of the curves at a true zero time, i.e., immediately after secretion, cannot be determined by the method employed, and since the decay is not first order, the slopes of tangents at any point vary with the period of the decay. For

these reasons, no reliable estimate of the quantitative influence of temperature on the rate of decay can be made from these data.

Pressure effects at low temperature. Figure 4 shows the changes in luminescent intensity, upon the sudden application and sudden release of pressure, during the course of decay at 10°. The application of pressure causes an immediate decrease in light intensity and the effect is reversible on release

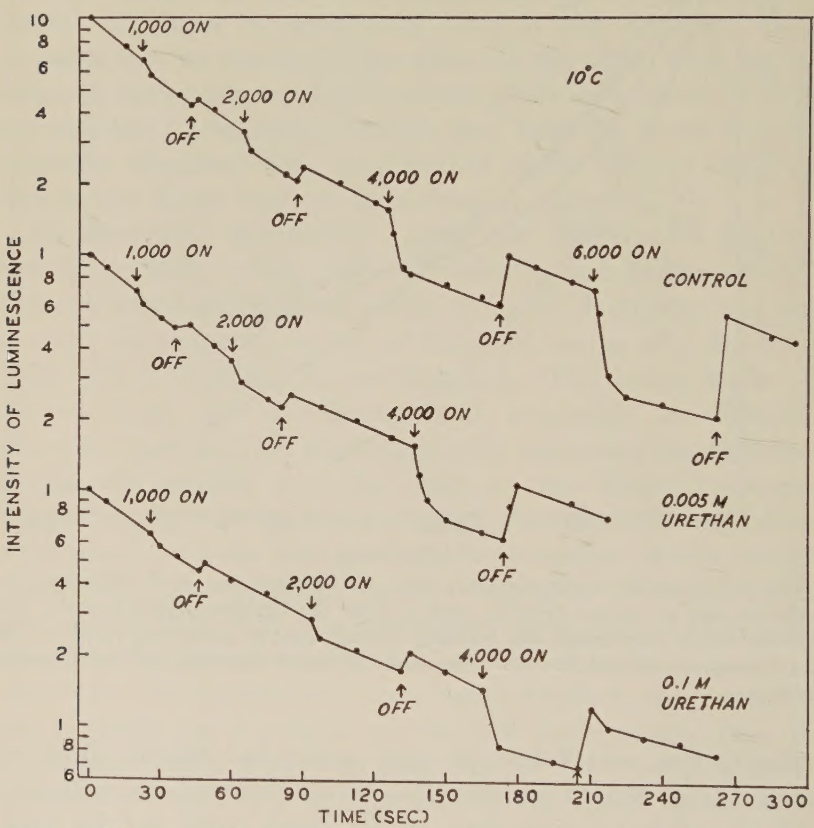

Fig. 4 Influence of changes in hydrostatic pressure (at points indicated in the figure, the pressure expressed as pounds per square inch) on the intensity of decaying luminescence in slime, with and without urethane, at 10°C. The three curves are plotted on the same time scale beginning at arbitrary points on the logarithmic scale of the ordinate. Differences in intensity of specimens containing urethane as compared to the control could not be determined in these experiments.

of pressure. Following the initial sudden drop under pressure, the luminescence continues to decay at the lower level of intensity. Although it is evident that the amount of the sudden changes in intensity is a function of the amount of pressure applied or released, the quantitative relationship is not a simple one, i.e., it is indicative of more than a single equilibrium or rate process in the total effect. The change in intensity resulting from application or release of 6,000 psi, would give a volume change of 160 cm³ per mole for the overall reaction computed from equation (1),

$$\Delta V = \frac{RT\ (\ln K_{p_1} - \ln K_{p_2})}{P_2 - P_1} \tag{1}$$

assuming that the observed effect is caused by the influence of a single equilibrium. The same value would result for $\Delta v^{\ddagger}$, computed from the analogous equation from the theory of absolute reaction rates (Eyring, '35; Glasstone, Laidler and Eyring ,'41), for a volume change of activation rather than of reaction.

Since both temperature and pressure have been found to modify the inhibitory action of urethane on bacterial luminescence (Johnson, Brown and Marsland, '42; Johnson, Eyring and Polissar, '54, p. 422), it is of interest to investigate the influence of pressure on *Chaetopterus* material in the presence of urethane at different temperatures. According to Johnson ('59), the inhibition of luminescence in slime of *Chaetopterus* by urethane is reversible by dilution. In the present study, the amount of reduction in light intensity by different concentrations of urethane was not determined. It was observed, however, that in the presence of 0.5 M urethane, almost complete inhibition took place. Since concentrations of about 0.1 M have been found to cause moderate inhibitions of luminescence in the slime secreted by the Hawaiian *Chaetopterus* (Johnson, '59), a concentration of 0.1 M, and a much lower concentration of 0.005 M, were used in the present study. At the relatively low temperature of 10°, no influence of 0.005 M urethane on the effect of pressure was evident.

However, with 0.1 M urethane, at 4,000 psi, a slight overshoot of light occurs on the release of pressure. A similar effect is much more pronounced at a somewhat higher temperature, as discussed below.

Pressure effects at room temperature. At 20°, low pressures, 600–1,000 psi, with or without urethane, exert no appreciable effect on the luminescence of slime (fig. 5), although these pressures cause a slight inhibition at the lower temperature of 10°. This result resembles the pressure effect on bacterial luminescence to the extent that the inhibitory effect of pressure is enhanced by low temperature.

At 2,000 psi, an immediate slight drop of light intensity is followed by a slight recovery, and the tendency to recover, under sustained pressure, is accentuated by the presence of 0.1 M urethane (fig. 5). Moreover, at this temperature, an overshoot of intensity is observed in specimens, with and without urethane, upon the release of 2,000 psi or a higher pressure.

Pressure effects at relatively high temperature. At 30° (fig. 6), the luminescent reaction is less sensitive to pressure than that at 10° or at 20°. Moreover, initially inhibitory effects are followed at once by a tendency to recover to the former level of intensity. The addition of urethane caused some decrease in the amount of inhibition, as well as an increase in tendency to recover, under the influence of pressure. At this temperature, a low concentration of 0.005 M urethane is sufficient to modify the effect of pressure, and the higher concentration of 0.1 M is so inhibitory that it was difficult to obtain the desired data. The pronounced increase in potency of urethane, with rise in temperature, resembles the effects of this drug on bacterial luminescence and other processes (Johnson et al., '54, pp. 408, 420, 421).

B. Notopodia

Response to a single stimulus. A single stimulus, of above threshold value, induces only a single flash from the excised notopodium. The luminescent response rises quickly to a

peak, and gradually decays over a period of a few seconds, depending upon the temperature. At temperatures below 14° or above 26°, a single shock, even of 250 volts, would evoke at best a very weak luminescent response, and usually none at all.

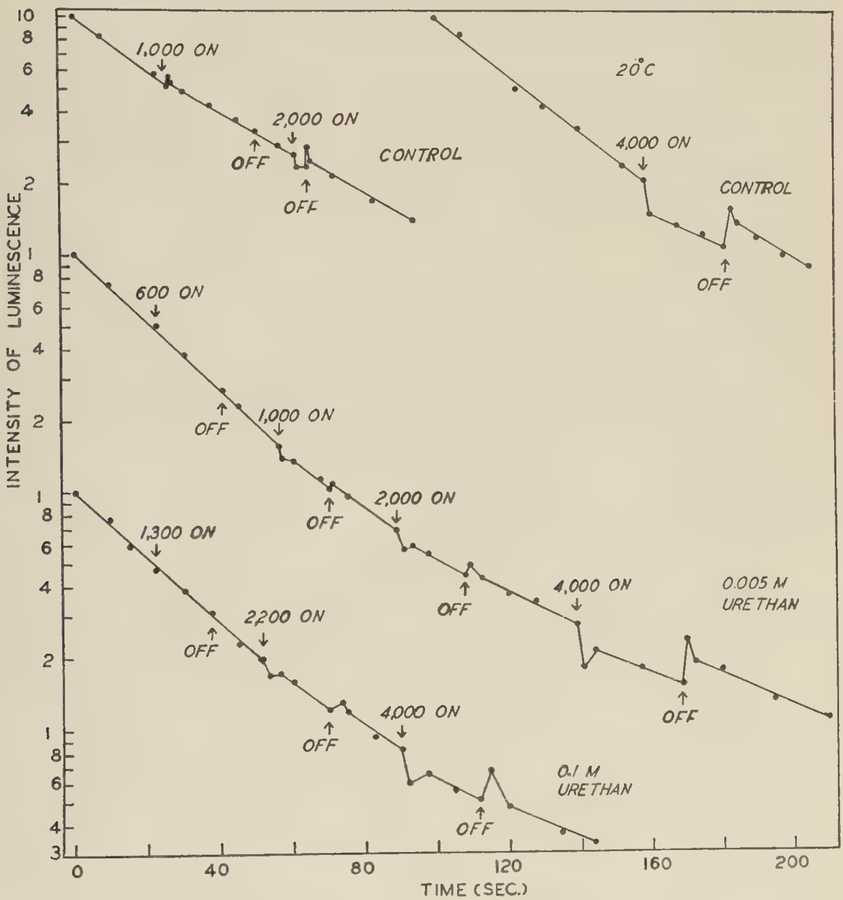

Fig. 5 Similar to figure 4, but at 20° instead of 10°C.

The excitatory process is evidently limited to a considerably narrower range of temperatures than that for luminescence of the free system once it has been secreted. Within the temperature range where a response will occur, single shocks of equal strength, separated by intervals of a few minutes, give

rise to successive responses characterized by a progressive, rapid decrease in maximum intensity.

The excitatory mechanism is not only more sensitive to temperature than is the process of luminescence itself in secreted

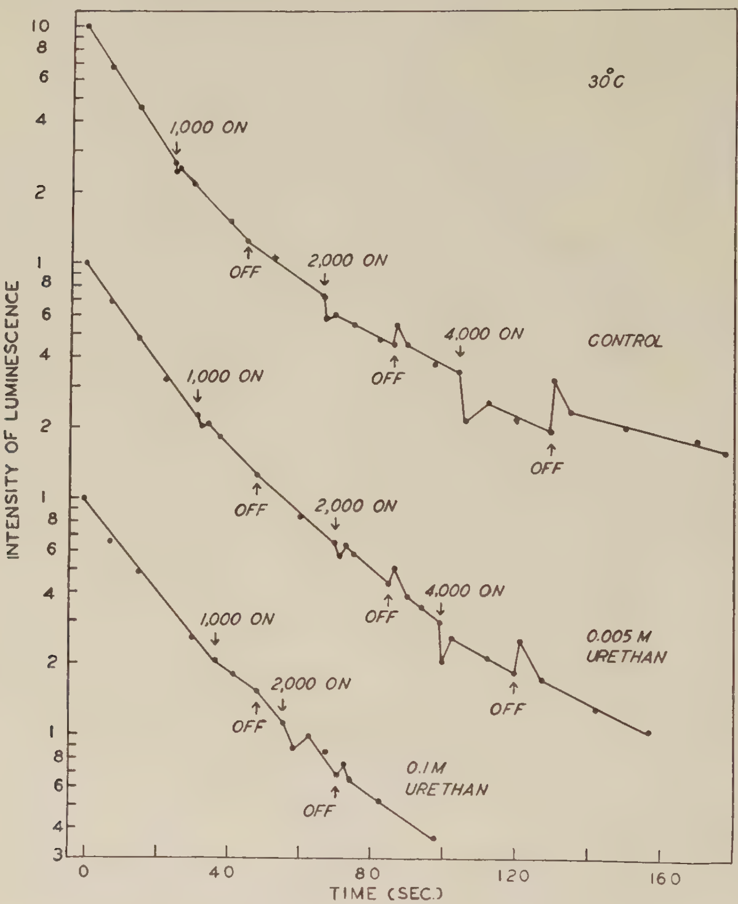

Fig. 6 Similar to figures 4 and 5, but at 30°C.

slime, but it is also much more sensitive to urethane. Thus, concentrations of urethane in excess of 0.005 M completely inhibit the luminescent response in excised notopodia.

Latent period. Photographic tracings of the available oscillograph records of single flashes in excised notopodia indi-

cate that the latent period is quite long in comparison to that of many other excitatory systems. Although precise data would require a more refined method, the present results indicate that, in the same specimen, the latent period varies somewhat beyond experimental error even at identical stimuli. At 22°C, it ranges between 0.35 and 0.4 seconds. In *Mnemiopsis,* the extreme range of the latent period for a single flash in a small piece of excised canal extends from 4.5 to 38 msec, depending on the temperature, strength of stimulus and individual specimen (Chang, '54). Nicol ('53) has reported a latent period of 18 to 20 msec for a single flash in *Acholoe* and 19 msec for a single flash in *Polynoe,* probably at room temperature.

The latent period in excised notopodia of *Chaetopterus* is lengthened by urethane, as well as by low temperature. At 22°, it ranges from 0.35 to 0.4 sec, while in 0.001 M urethane at the same temperature, it ranges from 0.4 to 0.55 sec. At a lower temperature, 15°, the latent period in absence of urethane, is increased to between 0.5 and 0.6 sec. At this temperature, the latent period appears to vary considerably under a constant moderate pressure of 1,000 psi, ranging between 0.45 and 0.7 sec.

Pressure effects on single flashes. The pressure effect on single flash responses at different temperatures, with and without urethane was studied. The observed effect depended somewhat on the time that pressure was applied; it was different when applied during the phase of rising intensity and at the time of maximum intensity respectively, but was essentially the same when applied at or subsequent to the peak intensity.

1. Pressure applied at the peak. Except for minor differences, as shown in figures 7, 8, 9 and 10, increased pressure at the peak of the flash results in a reduction of the light intensity to an extent that depends on the amount of pressure applied, either with or without urethane. Following the initial drop, the rate of decay under pressure becomes less. At 14° (figs. 7 and 8) and at 26° (fig. 10), a slight recovery of the

light intensity precedes this slower decay. The release of pressures of 2,000 to 4,000 psi is accompanied by a pronounced increase in light intensity at all temperatures. Urethane, in the concentrations studied, caused no obvious, qualitative change in the net effects of pressure.

2. Pressure applied during the ascending phase of the flash. At 15° and at 26°, during the rise of light intensity in a flash, a moderate pressure, such as 2,000 psi, causes a momentary decrease of light in specimens containing urethane (figs. 8*D* and 12*E*). The light then continues to increase, but at a slower rate than at normal pressure or in absence of

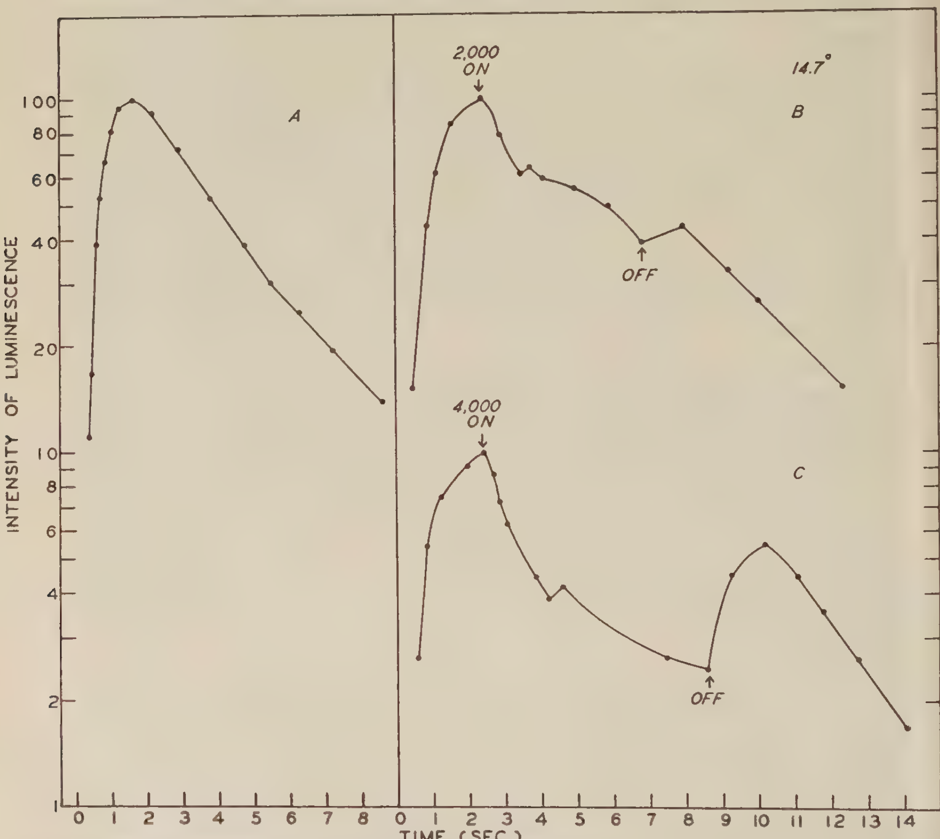

Fig. 7 Single flashes of excised notopodia at 14.7°C. Curve *A*, normal pressure throughout. Curves *B* and *C*, influence of applying pressures of 2,000 and 4,000 psi, respectively, at the flash peak, then releasing pressure a few seconds later.

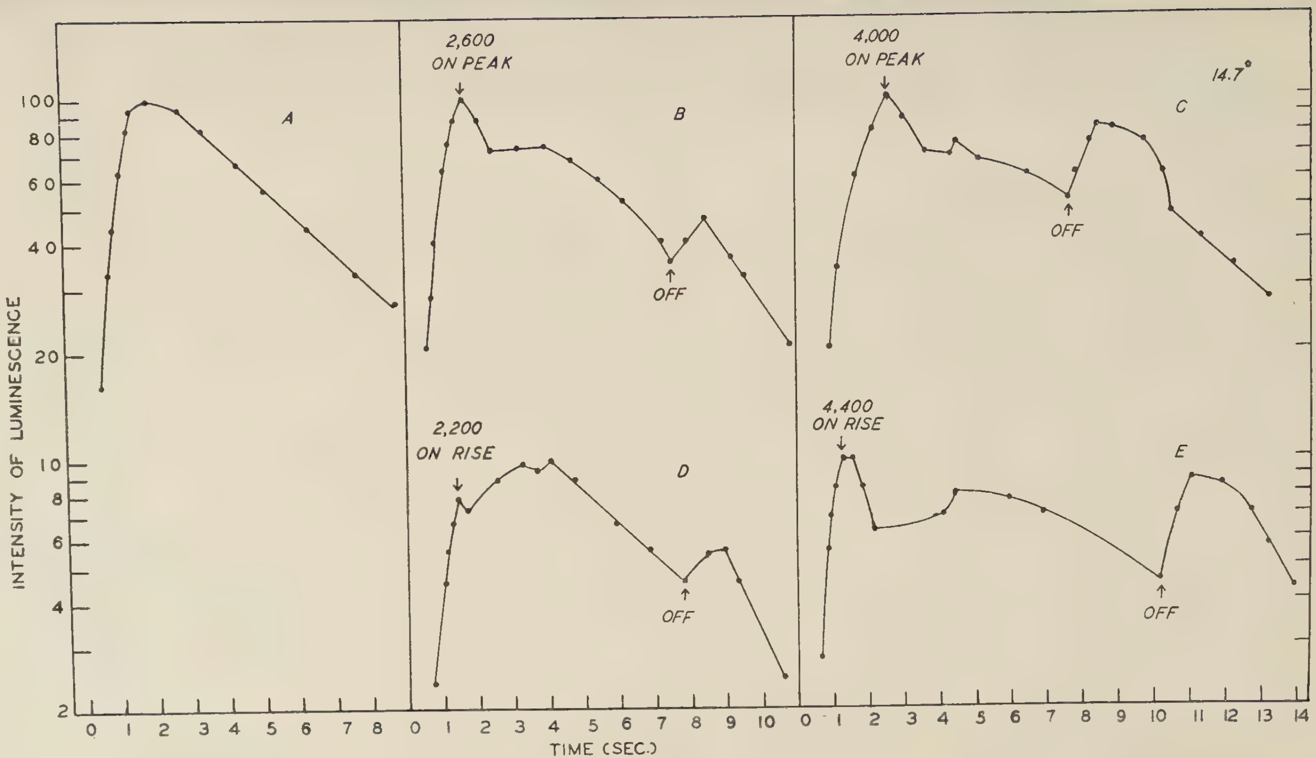

Fig. 8 Single flashes of excised notopodia in 0.005 M urethane, at 14.7°C. Curve *A*, normal pressure throughout. Curves *B* and *C*, increased pressures applied at the flash peak and released a few seconds later. Curves *D* and *E*, increased pressure applied during the ascending phase of the flash and released a few seconds later.

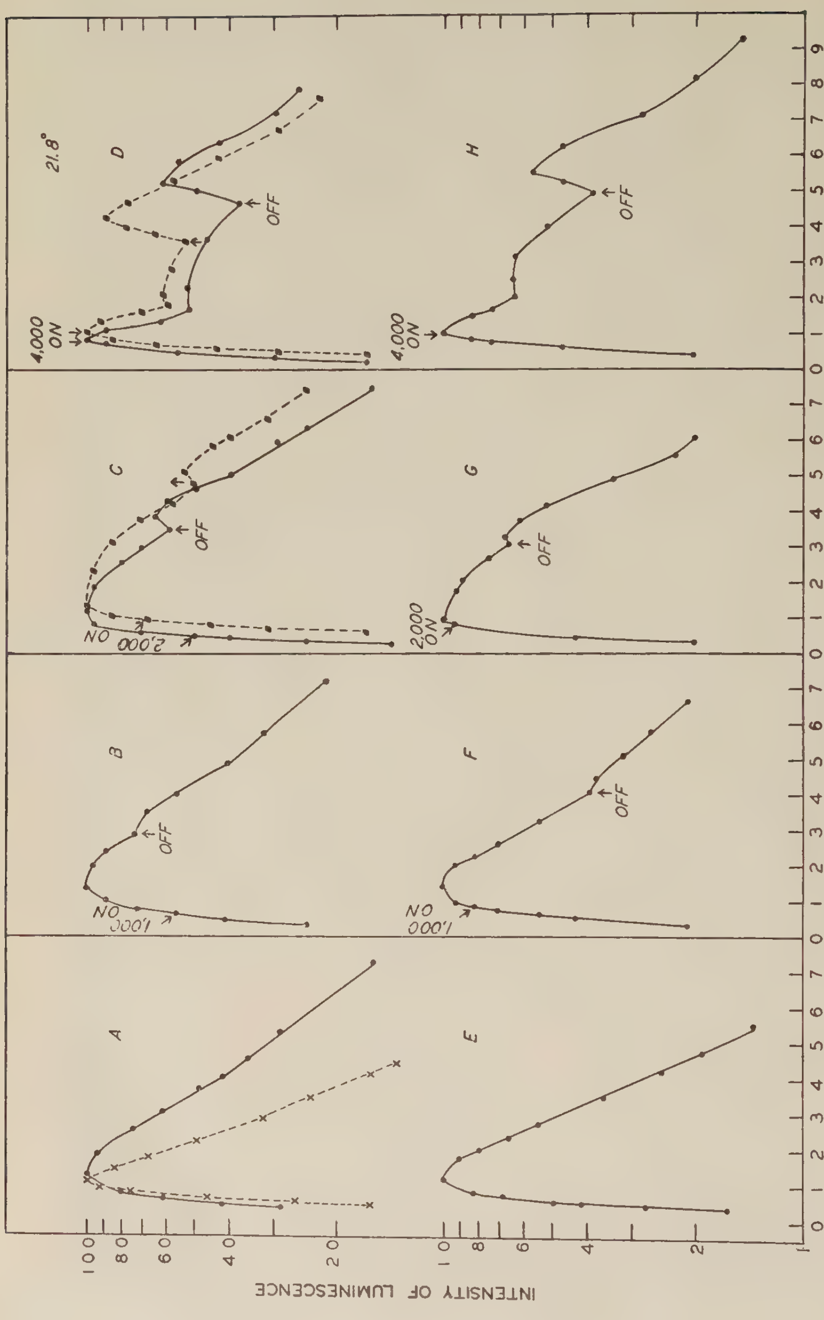

Fig. 9 Influence of applying and releasing various pressures, as indicated in the figure, on single flashes of excised notopodia at 21.8°, without urethane, (curves *A*, *B*, *C* and *D*) and in 0.001 M urethane (curves *E*, *F*, *G* and *H*). Pressure applied at the flash peak in each instance. Repeated experiments are indicated by the solid and broken lines, respectively, in *A*, *C* and *D*.

urethane. At room temperature, 21.8° (figs. 11*C*, *G*) but otherwise under the same conditions, the flashes from specimens, either with or without urethane, continue to rise without showing any apparent pressure effect. At 26°, and without urethane (fig. 12*B*) the same amount of pressure immediately reduces the light intensity with no evidence of a subsequent recovery under a sustained pressure.

The results of applying 4,000 psi are somewhat different from those just described for 2,000 psi. A pressure of 4,000 psi or above, causes an immediate decrease in light intensity, at all temperatures, with and without urethane. At 15° (fig.

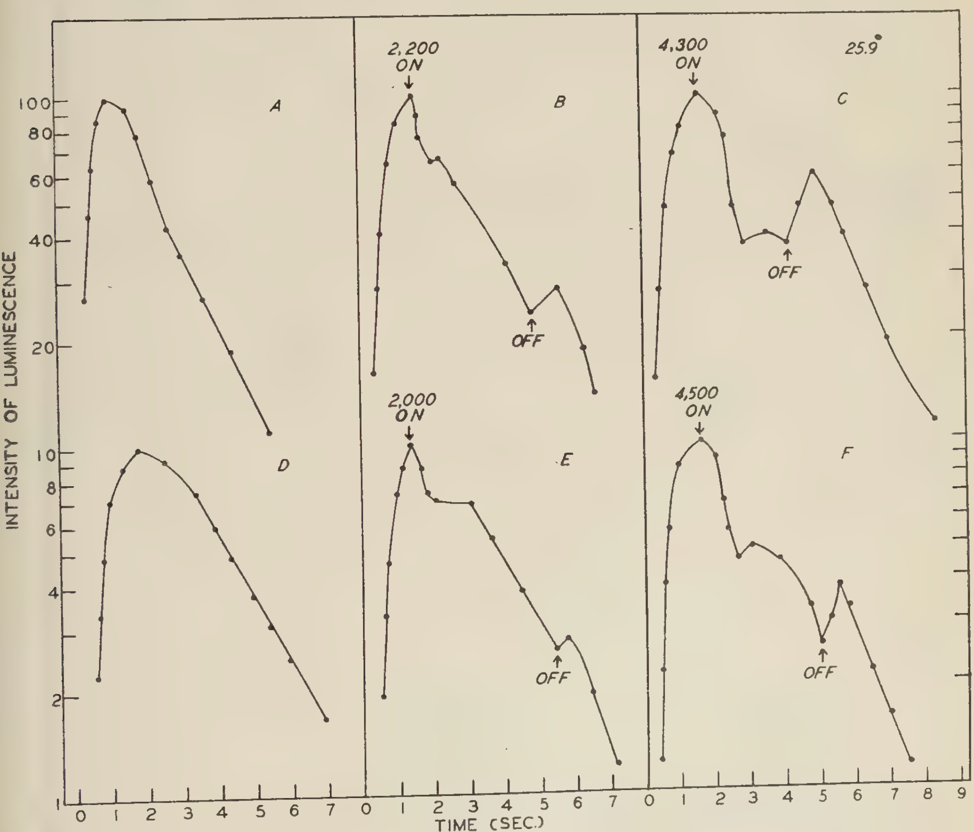

Fig. 10 Similar to figure 9, but at 25.9°C. Curves *A*, *B* and *C* without urethane; curves *D*, *E* and *F* with 0.001 M urethane.

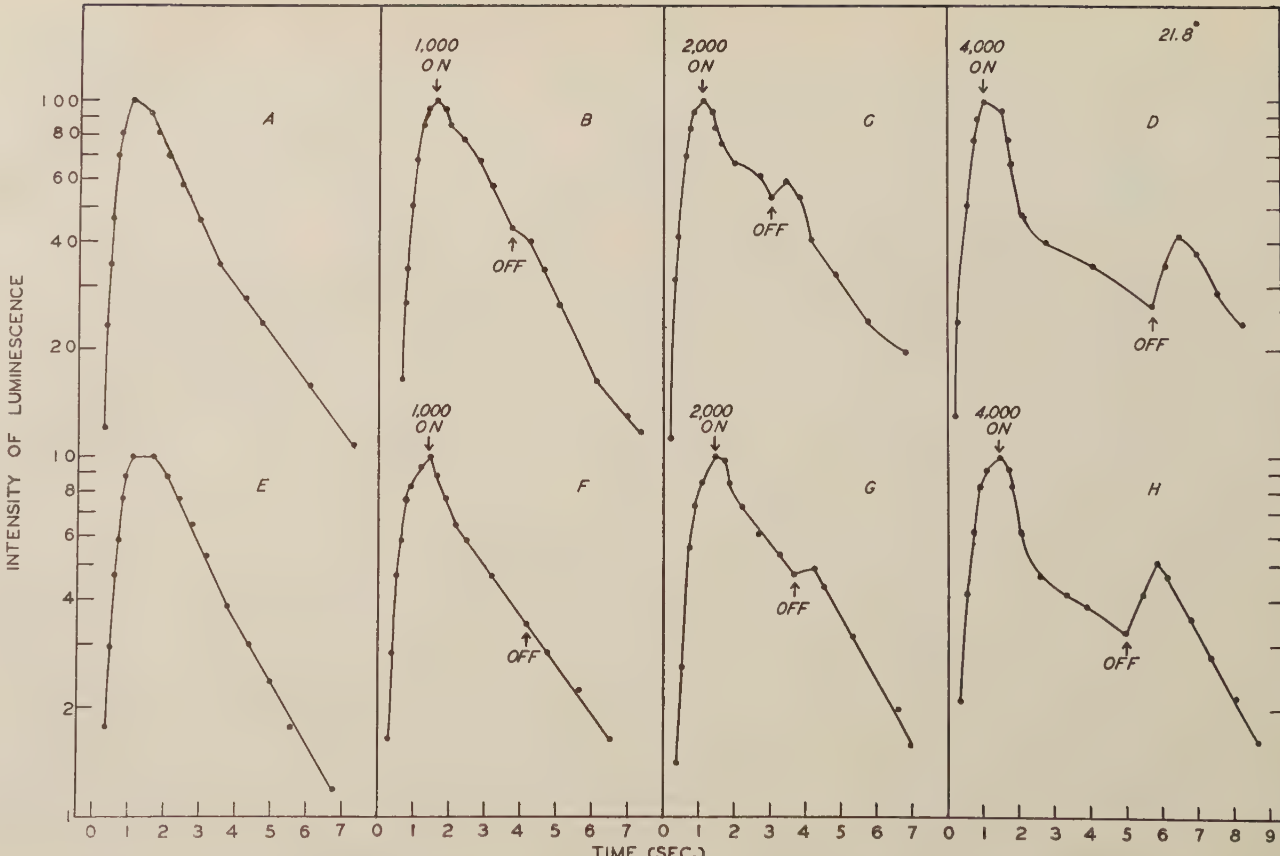

Fig. 11 Single flashes of excised notopodia at 20.8°C. Pressure was applied prior to the peak intensity. Curves *A*, *B*, *C* and *D* without urethane, and curves *E*, *F*, *G* and *H* with 0.001 M urethane.

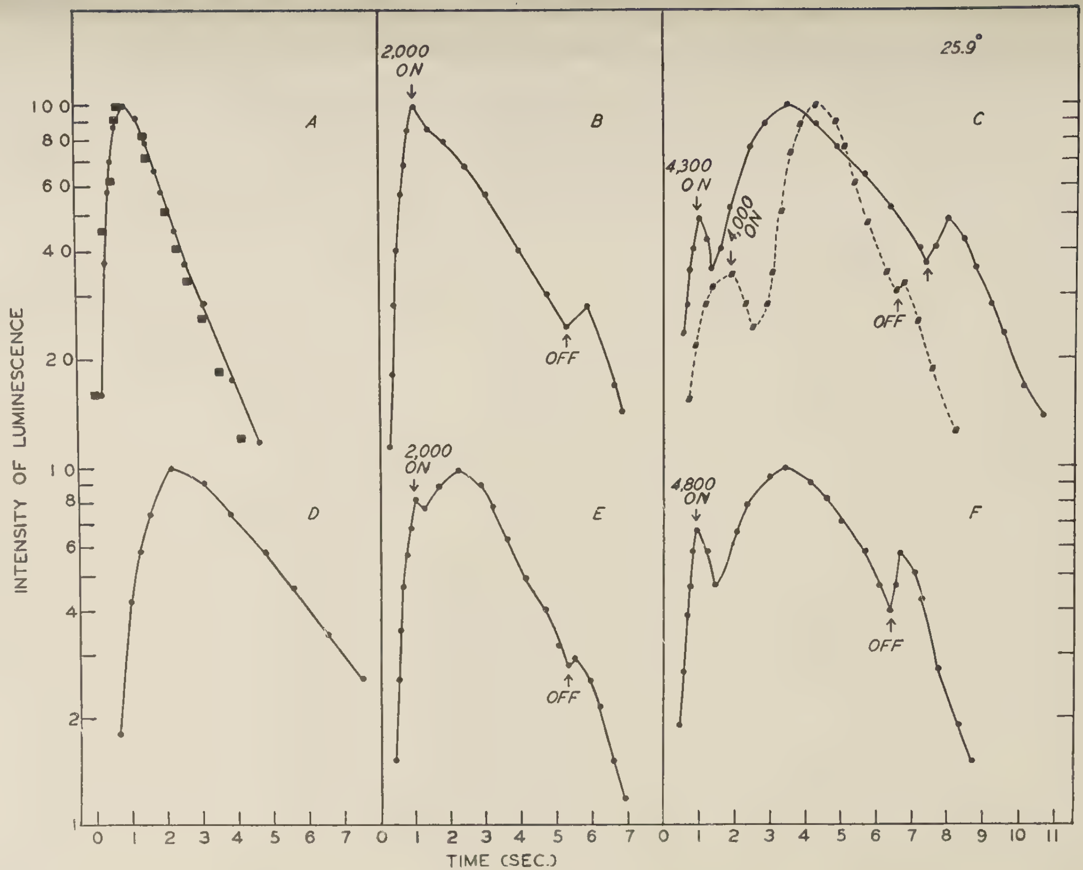

Fig. 12 Similar to figure 11, but at 25.8°C. The points on curve A are from two simultaneous records of the same flash recorded on the oscillograph (squares) and the Esterline-Angus (dots), respectively. Curves *A*, *B* and *C* without urethane; curves *D*, *E* and *F* with 0.001 M urethane. Repeated experiments shown in *C*.

8*E*) and 21° (fig. 12*C, F*), it not only recovers, but in addition it continues to rise to a maximum that is higher than the intensity at the time pressure was applied.

The transitory changes of the intensity of a single flash under increased pressure indicate that a multi-component system is involved in the luminescence of notopodia. In certain respects, the effect of pressure on the luminescent flash resembles its effect on muscular contraction. For example, in the sartorius muscle of the frog, at 0.5°, Brown ('36) found that the application of pressure of 3,000 psi during the course of contraction slows the contraction and depresses the tension to a lower value than in a control at normal pressure. If the pressure is applied at the peak of contraction, the tension is depressed abruptly to a lower value. A similar effect of pressure on slowing contraction and reducing tension in the retractor penis muscle of the turtle, at 20°, was also observed by Brown ('36), when pressure was applied during the course of the contraction phase. Furthermore, when applied at the peak of the contraction, pressure caused a rapid onset of relaxation. These effects apparently correspond to our result that a sharp decrease in light intensity is effected by pressure when applied at the peak of a flash.

Repetitive luminescent responses. Stimuli of equal strength and duration, repeated at appropriate intervals, evoke repetitive luminescent responses from a single notopodium, and these responses exhibit the phenomena of treppe and fatigue analogous to those observed by Chang ('54) for the rapid luminescent flashes in a small piece of Mnemiopsis canal. Nicol ('52b, '54b) observed a continuously increasing intensity of luminescence, which was interpreted as evidence of facilitation, during repeated stimulation of the aliform notopodia of whole *Chaetopterus* specimens, and this was followed by fatigue. Figure 13 shows a series of flashes resulting from repeated stimulation of an excised notopodium at 10-second intervals at room temperature. Treppe is evident in the first few flashes and continues, somewhat irregularly to a maxi-

mum flash intensity, followed by gradual fatigue. After each stimulation, at this frequency, the light does not return to complete darkness. Following the period of treppe, the fatigue is at first so gradual that an appreciable number of flashes of

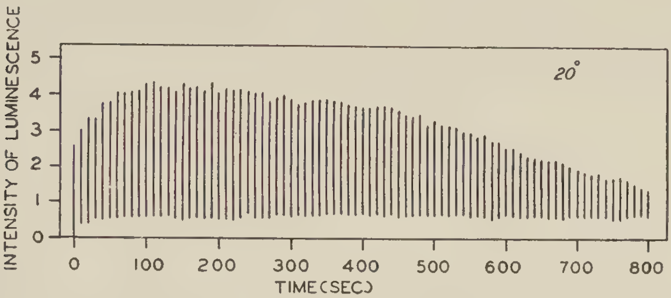

Fig. 13 Consecutive flash responses at 20°C, of excised notopodia to repeated, identical stimuli at 10 second intervals. The intensity and the duration of the stimuli are uniform. Sequence of flash heights, showing treppe and fatigue.

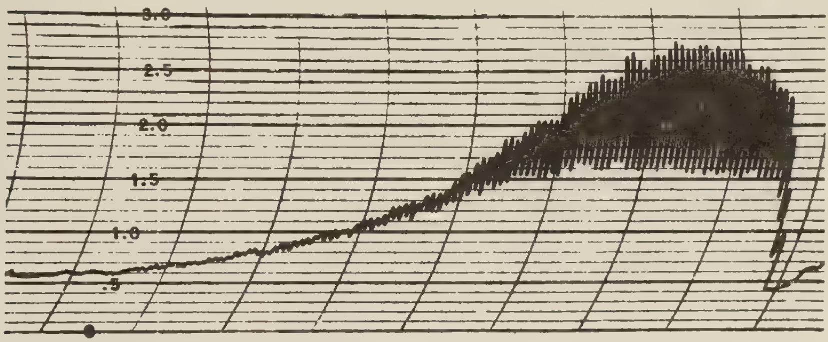

Fig. 14 Esterline-Angus record showing consecutive luminescent responses to repetitive stimuli at 2-second intervals, 21.5°C. Figure shows striking similarity to incomplete tetanus in muscle contraction. The vertical axis indicates luminescence intensity, with 0.4 as zero intensity. The horizontal axis represents time from right to left in units of 30 sec between successive lines.

almost uniform intensity results. With rapid stimulation, the same phenomena are observed over a shorter period of time, and the intensity of luminescence remains relatively high between the actual flashes (fig. 14). This response pattern is analogous to incomplete tetanus in muscle.

Effect of pressure on repetitive flashes. The pressure effect on repetitive flashes in specimens without urethane, at all temperatures, 14°–26°, is generally similar to the pressure effect on a single flash. When applied at the flash peak, pressure decreases the intensity of the following flash. Following the immediate decrease, the intensity of successive flashes under pressure increases to an extent that depends on the temperature, amount of pressure, and other conditions involved. Unlike the single luminescent response, the effect of pressure on repetitive luminescent responses is not only on the luminescent reaction per se, but also on the mechanism of excitation.

At low temperature, as shown in figure 16*A, B,* increased pressure causes a sustained, greater decrease of light intensity in the following flashes than at higher temperatures (figs. 15; 16*A, B*; 17*A, B*). Thus, at 14.8°, 6,000 psi practically abolishes the flash, whereas at 20°, the inhibitory effect under this pressure is less, and recovery under pressure takes place rapidly, reaching an intensity higher than just before the application of pressure. At 14.8°, upon releasing the pressure at the peak of a flash, the same flash becomes brighter, probably indicating an immediate increase in activity of the biochemical process of light emission, corresponding to the effect observed with the luminescent slime. An enormous increase of light in the following flash takes place, in this case possibly due to an influence on the excitatory mechanism, analogous to the augmentation in muscle tension resulting from application and release of pressure just prior to stimulation (Brown, '36).

At 21.5° (fig. 15), upon the release of pressure at the peak of a flash, no increase of light is observed in the same flash at 1,000 psi, but a considerable increase of light is noticed at higher pressures. A still larger increase of light occurs in the following flash, after which the successive flashes gradually diminish.

At 25.8°, the specimen becomes fatigued very rapidly under repeated stimulation (fig. 17*A, B*). At 2,000 psi, the decrease

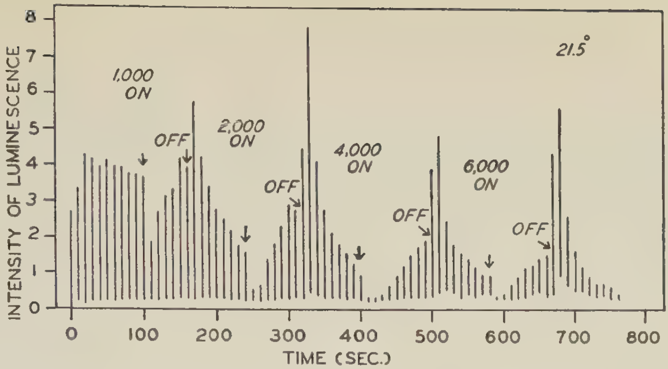

Fig. 15 Influence on flash heights, of applying and releasing pressure at the points indicated by the arrows; other conditions similar to figure 13.

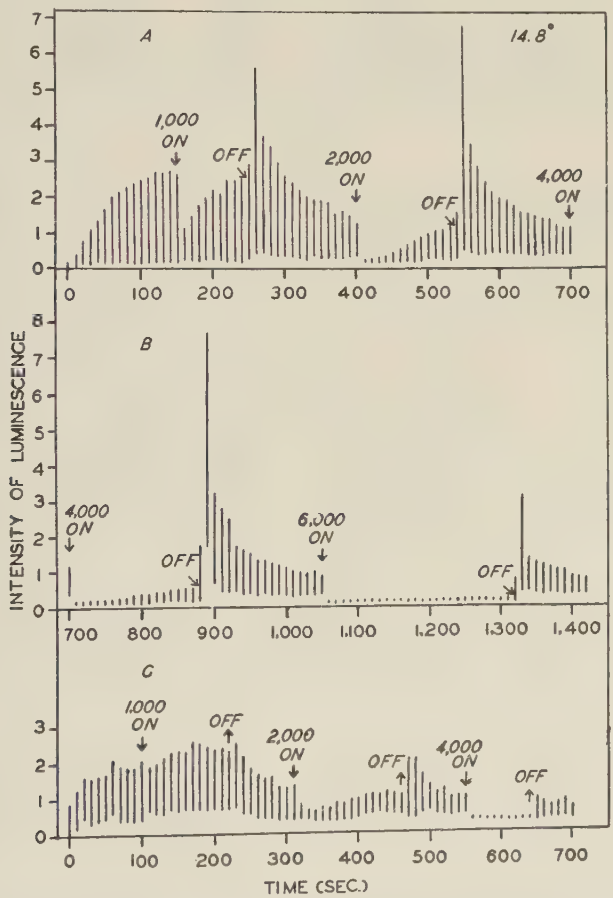

Fig. 16 Influence of various pressures, applied and released as indicated in the figure, on heights of consecutive flashes at 10 second intervals, 14.8°C. *A* and *B* represent one continuous sequence, without urethane; *C*, in 0.001 M urethane

of light intensity is less than that observed at the same pressure, at lower temperatures, i.e., 20°C or 14.8°C. Recovery under pressure does occur but is irregular. Similarly, inhibition of light at a higher pressure, 4,000 psi (fig. 17*B*), is slightly less than that caused by the same pressure at lower

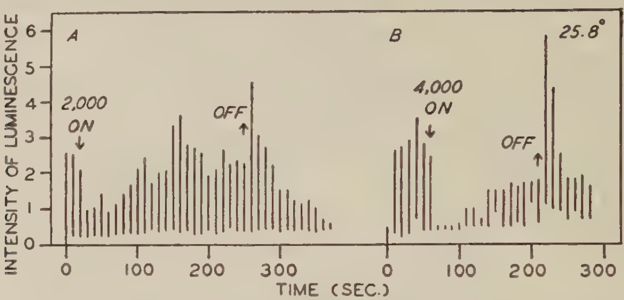

Fig. 17 Influence of applying and releasing pressures of 2,000 psi (*A*) and 4,000 psi (*B*) on consecutive flash heights. Stimulation at 10 second intervals; temperature 25.8°C.

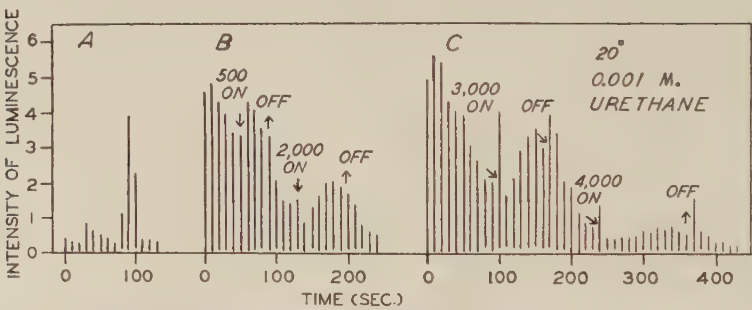

Fig. 18 Influence on consecutive flash heights of applying and releasing various pressures, at 20°C, the excised notopodia being in 0.001 M urethane. Stimulation once every 10 seconds, (A), and at 10-second intervals by three stimuli 0.1 second apart, (B) and (C).

temperatures. Little recovery of light under pressure is apparent at first, but an irregular recovery begins after a few abortive flashes. Moreover, when pressure is released at the peak of a flash, an increase in intensity occurs only in following flashes.

Effect of urethane. At 14.8°, in 0.001 M urethane (fig. 16*C*), the luminescent responses are weak under repeated stimula-

tion and are insensitive to the relatively low pressure of 1,000 psi. A somewhat higher pressure, 2,000 psi, causes an immediate decrease of light that is proportionally less than the decrease in a control without urethane. The smaller decrease of light intensity is accompanied by relatively little recovery under pressure. A pressure of 4,000 psi practically stops the light production and no appreciable recovery follows under pressure. An increased intensity of light occurs in the flash following release of pressure.

At 20° (fig. 18*A*), an irregular pattern of flashes is obtained in specimens with urethane, when stimulated at the rate of one shock every 10 seconds. A "normal" response pattern is obtained, however, when trains of 3 stimuli at the rate of 10 per second are given at 10 second intervals (fig. 18*B, C*).

Under these conditions the effect of pressure is rather complicated. Thus, a low pressure, 500 psi, increases the flash intensity and this effect is reversible. To the same specimen, application of 2,000 psi reduces the light intensity in the following flash; a rapid recovery then ensues, proceeding to even higher intensity in the successive flashes. When the pressure is released, the following flash is brighter. This is followed by a sharp decrease in the next flash, after which the light continues to decrease gradually in the successive flashes.

DISCUSSION

The present study reveals that both the biochemical system responsible for luminescence in the slime secreted from the aliform notopodia, and the physiological system responsible for the luminescent responses in excised posterior notopodia of *Chaetopterus* are not only sensitive to temperature, but also are remarkably sensitive to pressure. Throughout the temperature range studied, 10°–30°, the immediate effect of increased pressure is a decrease of light intensity, and this effect is reversible on the release of pressure. Moreover, the inhibitory effect of pressure is more pronounced at low temperatures (10°–15°) than at higher temperatures (20°–30°).

Under sustained pressure, the light intensity may or may not recover to a certain extent depending upon the temperature and other conditions involved.

The time course of changes in light intensity under the influence of pressure on either the secreted slime or the excised organs, indicate that more than a single equilibrium or rate process is affected. The present data, however, are not adequate to distinguish the actual number or types of reactions involved. It is reasonable to assume that the recovery process under pressure in slime is due to slow changes in steady states of sequential reactions. Additional reactions, no doubt involving the excitatory mechanism, participate in notopodial flashes. Examples of biological activities and biochemical reactions that depend on sequential and simultaneous reactions are numerous, and in a few instances the effects of pressure have been partially analyzed, e.g., bacterial luminescence (Johnson et al., '54, pp. 309–316; Strehler and Johnson, '54), ciliary beat (Johnson et al., '54, pp. 323–324; data of Pease and Kitching, '39), cell division (Marsland, '57) and muscular contraction (Brown, '57).

In the cell free and partially purified system of *Cypridina* the intensity and rate of decay of luminescence are practically unaffected by pressures of 2,500–5,000 psi, although these pressures cause a slight, reversible increase in intensity when the reaction mixture contains some reversibly oxidized luciferin (Bronk, Harvey and Johnson, '52). Luminescence in the slime of *Chaetopterus,* on the other hand, is strongly inhibited by these pressures, and in this respect is similar to bacterial luminescence at temperatures below the optimum (Johnson et al., '54; Strehler and Johnson, '54). Strongly inhibitory effects of such pressures have been observed also, under certain conditions, in the "squeezates" of Mnemiopsis (Chang and Johnson, '59). In the luminescent slime of *Chaetopterus,* the inhibitory effect of a given pressure is less at high temperatures, as in bacteria, but unlike bacteria, no actual increase of light intensity, due to pressure, is observed

within the limit of high temperatures (20°–30°C) which could be studied by the methods at hand.

As noted in a preceding section, the inhibitory action of urethane on luminescence is much more pronounced in notopodial flashes than in free slime, indicating a greater sensitivity of the excitatory mechanism than of the light-emitting process itself to this drug. Urethane somewhat modifies the effects of pressure on notopodial flashes, in a complicated way. In the luminescence of slime also, urethane influences the net result of increased pressure according to somewhat complex inter-relations between drug concentration, temperature and amount of pressure. At a low temperature, no perceptible influence was detected within the range of the variables investigated, but at 20° to 30°C the following relations appeared. The higher of the two concentrations studied, 0.1 and 0.005 M respectively, prevented the decrease in light intensity normally caused by a low pressure, i.e., ca. 1,000 psi, and made the inhibitory effects of higher pressures less pronounced. The influence of the lower concentration was in the same direction, but to a less extent. The simplest interpretation is that, as in bacterial luminescence (Johnson et al., '54, p. 422; Strehler and Johnson, '54), as well as in the narcosis of tadpoles (Johnson and Flagler, '51) and of single nerve fibers (Tasaki and Spyropoulos, '57), the inhibitory action of urethane is subject to reversal by pressure, and that in the present instance, a similar reversal occurs concomitantly with the inhibitory action of pressure on the luminescent reaction directly. Thus in the presence of urethane, the net change in intensity is relatively small.

The effect of pressure on the luminescent responses of the excised notopodia bears resemblance, in some respects, to the compression of muscle (Brown, '36). Compression either at or after the peak of muscular contraction, causes a rapid reduction in tension. Similarly, compression of the notopodium at the peak of a flash causes a quick drop in luminescence intensity, though this is followed by more complicated changes than are apparent in muscle tension. Moreover, a

brief application, then release, of pressure just prior to, or overlapping with, the stimulation of muscle causes an augmentation of the twitch tension, and this phenomenon is possibly related, at least in part, to the increased luminescence intensity that always resulted in the next flash following release of pressure. Compression during the subsequent contraction of muscle, slows the contraction and depresses the tension; similarly, compression during the ascending phase of a single luminescent response, causes either a momentary decrease of light intensity or a slower rate of rise. Again resembling muscular contraction, and also the rapid luminescent flashes in *Mnemiopsis* (Chang, '54), the multiple flash responses in an excised notopodium, under repeated and identical stimulation, exhibit the phenomena of treppe and fatigue.

These several similarities in the behavior of muscle and notopodial luminescence, however, do not necessarily mean that muscle activity is actually involved in the notopodial flashes. On the contrary, they may be indicative of a fundamental similarity in the excitatory mechanism leading to muscle contraction and to the luminescent response, respectively. Consistent with this view is the evidence derived from a study of the adult firefly, *Photuris*, and its larval form, by Chang ('56), namely, the observation of treppe and summation of luminescent flashes in response to repeated, identical stimuli in the isolated photogenic organs, with which no muscle fibers are associated.

SUMMARY

A study has been made of the influence of hydrostatic pressure, temperature and urethane on luminescent flashes caused by electrical stimulation of excised, posterior notopodia of *Chaetopterus variopedatus*, and of the kinetics of decaying luminescence in the slime secreted from aliform notopodia. In notopodia, stimulation of single flashes is not "all-or-none;" the response varies with the strength of stimulus and with individual notopodial specimens. Adequate identical

stimuli elicit a consecutive series of flashes exhibiting treppe and fatigue.

The time course of a single flash is relatively slow at room temperature, lasting over a period of about 6–10 seconds, and the latent period is relatively long, lasting as much as 0.4 seconds. Both the duration of the flash and the latent period are longer at low temperatures, or in the presence of urethane.

Increased hydrostatic pressure reversibly inhibits the intensity of luminescence in both the notopodia and slime, to an extent depending on temperature as well as on the amount of pressure.

At normal pressure and temperatures of 10°, 20°, or 30°C the rate of decay of light in the luminescence of slime is not first order but decreases with time. In notopodia the luminescent flash response occurs only over a narrower range of temperature, from about 14° to 26°. At all temperatures, in either notopodia or slime, pressure causes an immediate and reversible decrease in the light intensity, and this effect is more pronounced at the lower than at the higher temperatures.

In free slime, urethane (0.005 to 0.1 M) reduces the inhibition of light intensity by pressure and enhances both the recovery and the after-pressure overshoot at 20°–30°C. The only effect of urethane (0.1 M) observed at 10° was a slight overshoot of light intensity on release of pressure.

In single notopodial flashes, at temperatures between 14° and 26°, increased pressures of 1,000 to 4,000 psi, when applied at the peak of a flash, cause a sharp decrease in light intensity. This is followed by a luminescent decay at a slower rate. Light intensity then increases again on release of pressure. When pressure is applied during the ascending phase of a single flash, variable and complicated changes occur. In single notopodial flashes, no clear influence of urethane on the effects of pressure could be discerned under the conditions studied. The luminescent flash of notopodia is found to be much more sensitive to urethane than is the intensity of luminescence in the slime. With the former, 0.005 M urethane is the highest concentration in which a satisfactory

response occurs. Moreover, in both slime and notopodia, the pressure effect is more pronounced at 10° to 14° than at 20° to 30°. At 14°, urethane weakens the luminescent response and reduces the inhibition caused by pressures of 2,000 psi or less.

The results are discussed briefly with reference to interrelations in the influence of pressure, temperature and urethane on different types of luminescent and other systems, and with reference to some fundamental resemblances in the effects of these variables on luminescent flashes and muscular contraction, respectively.

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THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID DURING EARLY EMBRYONIC DEVELOPMENT OF RANA PIPIENS [1]

PHILIP GRANT [2]
The Institute for Cancer Research, Philadelphia, Pennsylvania

THREE FIGURES

INTRODUCTION

Discussion as to whether a "partial" or "total" synthesis of deoxyribonucleic acid (DNA) occurs during amphibian development has recently been renewed (Brachet, '54; Lovtrup, '55). The application of more sensitive methods to the determination of DNA, particularly the microbiological assay procedure of Hoff-Jorgensen ('51), has revealed the existence of quantities of DNA in eggs and embryos of amphibians far in excess of that to be expected, assuming the constancy of DNA per diploid nucleus. This has been demonstrated in several different amphibian species in which a variety of analytical methods have been employed (Hoff-Jorgensen and Zeuthen, '52; Hoff-Jorgensen, '54; Brachet, '54; Sze, '53; Gregg and Lovtrup, '55; Lovtrup, '55). Values range from 2,700 times the diploid value in *Rana platyrrhina* to 95,000 times the diploid value in *Rana pipiens*.

Although there is agreement as to the presence of excess amounts of DNA, there is conflicting evidence concerning the

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[2] Present address: Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland.

amount of this excess and the pattern of synthesis of DNA during early cleavage. Brachet ('54) and Sze ('53), using conventional analytical procedures, have reported a gradual net synthesis of DNA during cleavage followed by a rapid linear increase after the blastula stage. However, the group using the microbiological assay (Hoff-Jorgensen and Zeuthen, '52; Lovtrup, '55; Gregg and Lovtrup, '55) report a constant amount of DNA per embryo during cleavage until the late blastula stage. To explain this pattern of synthesis, it has been suggested that the excess DNA, presumably located in the cytoplasm, serves as a precursor reserve for the rapid synthesis of nuclear DNA during cleavage ("partial" synthesis). After its exhaustion at the blastula, a "total" synthesis of DNA is initiated to account for the linear increase observed.

The experiments to be reported here were designed as an attempt to evaluate the conflicting evidence mentioned above. A modification of the Hoff-Jorgensen microbiological assay procedure for the determination of DNA and free deoxyribonucleosides was employed. However, rather than analyze acetone-dried whole embryos, analyses were made on cold acid soluble extracts of homogenized embryos to determine the levels of acid soluble deoxyribonucleosides and deoxyribonucleotides. The cold acid-insoluble residue was then analyzed for DNA according to the procedure of Hoff-Jorgensen. Thus, at any one stage of development, values for the free and bound deoxyribonucleosides were distinguishable. By contrast, the determinations of Hoff-Jorgensen do not permit a clear distinction between free (acid-soluble) and polynucleotide bound (acid-insoluble) deoxyribonucleosides.

MATERIALS AND METHODS

Eggs were obtained from *Rana pipiens* females by the usual methods of induced ovulation. Batches of eggs were fertilized, separated into finger bowls, each of which contained approximately 100 eggs, and allowed to develop at room temperature, (17–20°C) or at 15°C. At appropriate intervals, 25–75 em-

bryos were taken for analysis. The jelly was removed and the embryos were washed three times in 10% Holtfreter's solution. They were then transferred to a 12 ml centrifuge tube and the excess solution was pipetted off. The tube was then plunged into a dry ice-dowanol freezing mixture (—75°C) for 5 minutes and stored in a deep freeze (—40°C) until a complete developmental series had been collected. Usually 15–20 developmental stages were collected.

Extraction of the embryos proceeded as follows: The frozen embryos were brought into a 4°C cold room and allowed to stand for a few minutes. To each tube, 0.5 ml of ice cold 2% perchloric acid was added and the embryos were homogenized with a stirring rod. The tubes were then placed in an ice bath for 5–10 min. with occasional stirring. An additional 0.5–1.0 ml of cold 1% perchloric acid was added to make the final volume approximately 1–2 ml. The mixture was stirred vigorously and centrifuged at 2500 rpm for 15–20 min. in a refrigerated International Centrifuge, Model PR 1. This cold acid extraction was repeated two times with 0.5–1.0 ml each of cold 1% perchloric acid and the supernatants were pooled. This represented the acid soluble extract. It was neutralized by the addition of 0.07–1.0 ml of 5 N ammonium hydroxide. It was then extracted two times with chloroform to remove any protein and pigment which may have been carried over. The chloroform was removed by placing the tubes in a vacuum desiccator. The extract was then ready for microbiological assay.

The acid-insoluble residues were extracted once with cold 95% alcohol, two times with boiling alcohol: ether (3:1) and once with acetone and allowed to dry overnight in a vacuum desiccator. To the dried residues, 1.0–1.5 ml of 0.5 N NaOH was added and the mixture was stirred vigorously to homogenize. The tubes were placed in a boiling water bath for 15 min. and were stirred occasionally to effect a maximum dissolution of the residue. After cooling, an 0.5 ml aliquot was pipetted into a 15 ml centrifuge tube, to which was then added 2.5 ml of a buffer-activator solution (0.06 M maleic

acid; 0.01 M $MgSO_4$). The pH was adjusted to 6.5–7.0 with a few drops of 0.5 N NaOH. Finally, 0.1 ml each of a freshly prepared DNAase solution (1 mg/ml) and snake venom phosphatase (0.2 mg/ml), were added and the mixture allowed to incubate at 37°C for 20–24 hours. The tubes were then centrifuged and the supernatant solutions made up to a 5 ml volume. Aliquots of 0.1–1.0 ml (depending on stage of development) were taken for assay.

The assay procedure is a modification of Hoff-Jorgensen's ('51) assay method using *Thermobacterium acidophilus R 26.* It was developed by Travaglini (see Schultz, '56) and employs a double strength basal medium with the addition of folic acid (1.05 mg/100 ml) and Tween (1 ml 10% Tween 80 to 100 ml of medium) (Toennies et al., '52). This medium contains uracil (0.02 mg/ml) so as to eliminate one of the objections raised by Siedler et al. ('57) concerning the original basal medium employed by Hoff-Jorgensen. They found that the addition of uracil and/or enzyme-digested casein would stimulate the growth of the organism in the presence of thymidine.

Thymidine was used as a standard and incubation was carried out at 37°C for 18 hours. Optical densities were read at 650 mμ in a Model DU Beckman spectrophotometer. A standard thymidine curve was run simultaneously with each determination and the values, both for the free deoxyribonucleosides and for DNA, were calculated from the curve. One gm mol deoxyriboside is equivalent to 327 gm DNA.

Several experiments were carried out in which solutions of highly polymerized calf thymus DNA were carried through the alkali and enzyme digestion procedure as described above. The best determinations yielded approximately 80% total hydrolysis when compared to theoretical thymidine release. The poorest resulted in 40–50% hydrolysis. Whether this could be attributed to the highly polymerized state of the DNA standard or to other factors was difficult to determine. The digestion of the embryonic material gave consistently reproducible results. Since the conditions under which the

embryo DNA was hydrolyzed were different from those employed with the standard DNA (high protein content of the embryo extract, acid and lipid extractions which probably altered the nature of the embryo DNA), it was considered desirable to use the thymidine standard for calculations of DNA. This is essentially the same procedure employed in the original method of Hoff-Jorgensen.

Although the evidence in the literature indicates that crude snake venom phosphatase and DNAase will hydrolyze DNA to nucleosides (Schmidt, '57), some recent work suggests an explanation for the failure to obtain complete deoxyriboside activity from the enzymic digestion of standard DNA. Evidently, one possibility is the failure to obtain complete DNAase digestion of the DNA to a mixture of oligonucleotides, di- and tri-nucleotides, thus leaving a core of undigested DNA. This mixture may be partly hydrolyzed to a mixture of nucleosides and nucleotides by the snake venom phosphatase. Schneider and Potter ('57) have shown that *Thermobacterium acidophilus R 26* grows very poorly on di- and tri-nucleotides; moreover, Siedler et al. ('57) have shown that there is a differential response to mononucleotides. Deoxyguanylic and thymidylic acids, for example, are only 11–22% as active in producing growth as is thymidine. Thus, in addition to the possibility of failure to digest all the polynucleotide to simpler nucleotides and nucleosides, leaving an undigested core of DNA, the double digestion may result in a mixture of nucleosides with some mono-, di- and possibly tri-nucleotides which are relatively less active than nucleosides.

RESULTS

In table 1, the values for micrograms of acid soluble "free" deoxyribonucleosides and/or deoxyribonucleotides per embryo are shown for developmental stages at two temperatures. The ranges of values are also shown at each stage. The values at the higher temperatures are generally greater, particularly during the first 18 hours. From 20 hours, however, the values at the two temperatures are essentially similar. It has not

TABLE 1

The levels of acid soluble deoxyribonucleosides and/or deoxyribonucleotides per embryo during development

STAGE	TEMPERATURE							
	15°				17–20°			
	Hours	No. Exps.	μg/emb.	Range	Hours	No. Exps.	μg/emb.	Range
1	0–1	3	0.020	(.016–.027)				
2	2	6	0.018	(.014–.024)	2	4	0.024	(.006–.038)
4	6–8	4	0.020	(.013–.028)				
6					5– 8	3	0.053	(.023–.085)
7–8⁻	11–13	3	0.040	(.016–.052)				
8⁻					9–12	4	0.066	(.036–.080)
8⁻–8	18	3	0.020	(.011–.034)	13–17	4	0.063	(.021–.109)
8					18–20	3	0.015	(.014–.017)
8–8⁺	23–25	3	0.013	(.088–.022)				
9	27–31	3	0.013	(.007–.022)	21–25	3	0.019	(.011–.025)
10	36–41	5	0.015	(.008–.023)	26–31	4	0.015	(.007–.022)
11	42–46	4	0.011	(.007–.013)	32–36	4	0.016	(.010–.019)
12	55–65	4	0.013	(.010–.016)	40–48	3	0.016	(.013–.021)
13	72	6	0.012	(.006–.020)	52	2	0.017	(.013–.021)
14–15	90–100	4	0.012	(.011–.013)				
16	105	3	0.013	(.008–.020)				
17	120	5	0.011	(.007–.022)				
18	150	5	0.019	(.013–.029)				
19	170	4	0.025	(.015–.039)				
19–20	185	2	0.033	(.021–.044)				
20	218	2	0.025	(.019–.030)				

been ascertained whether the differences observed at the two temperatures are significant.

The unfertilized egg contains 0.020 μg of acid soluble deoxyriboside activity (15°C). During the first 12 hours of early cleavage, there is an intense synthesis of deoxyribosides. The value at stage $7\text{–}8^-$ is significantly different from the values at stage 4 or stage $8^-\text{–}8$ ($P < 0.05$). This is much more evident at the higher temperatures which indicate almost a threefold increase in 12 hours. During the subsequent 8 hours, until a mid blastula stage (stage 8), the levels of free deoxyribosides have fallen to approximately one-fourth to one-fifth the amount present at very early blastula when the cell number was approximately 64–100 cells. During gastrulation and neurulation to the tailbud stage, the levels are constant. Beyond tailbud, until hatching (stage 20), there is evidence of a renewed synthesis.

The synthesis of DNA is shown in table 2. Here again, values at the higher temperatures are greater than those at 15°C for corresponding stages. One factor which may be responsible is the error in accurately determining the stage. Since the rate of development at the higher temperatures is greater, a compression of stages results, particularly in early cleavage. However, the fact that higher values at 17–20° are consistent throughout development suggests that there may be a significant difference at the two temperatures. The evidence is insufficient, however, to permit any definite conclusions.

The data indicate that under the conditions of analysis employed in these experiments, there is a net increase in the content of DNA per embryo during segmentation. Once again, this is considerably more evident at 17–20° than at 15°. Although the values between 2 and 12 hours (15°) are not significantly different, the value at 18 hours (stage 8^-) is significant ($P < 0.05$). At 17–20°, the value at stage 8^- is also significantly greater than the earlier values ($P < 0.05$). Thus, there is a relatively brief period (until a very early blastula of approximately 700–800 cells) when there seems to be a

TABLE 2

Synthesis of deoxyribonucleic acid during development

STAGE	TEMPERATURE 15° Hours	No. Exps.	µg/emb.	Range	TEMPERATURE 17–20° Hours	No. Exps.	µg/emb.	Range
1	0–1	4	0.022	(.012–.034)				
2	2	7	0.023	(.015–.038)	2	3	0.024	(.020–.028)
4	6–8	5	0.025	(.020–.039)				
6					5–8	3	0.031	(.023–.047)
7–8⁻	11–13	4	0.032	(.011–.044)				
8⁻					9–12	4	0.042	(.037–.047)
8⁻–8	18	4	0.041	(.029–.051)	13–17	4	0.108	(.063–.162)
8	22	1	0.102		18–20	3	0.228	(.191–.282)
8–8⁺	23–25	4	0.164	(.115–.234)				
9	27–31	4	0.267	(.163–.465)	21–25	4	0.408	(.298–.521)
10	36–41	5	0.563	(.470–.717)	26–31	3	0.689	(.660–.706)
11	42–46	2	0.734	(.600–.867)	32–36	4	1.00	(.795–1.28)
12⁻	55–60	6	1.11	(.761–1.37)				
12⁺					40–48	4	1.47	(1.32–1.58)
13	72	5	1.57	(1.47–1.77)	52	2	2.27	(2.25–2.28)
14–15	90–100	6	1.87	(1.03–2.55)	60	2	2.47	(2.36–2.57)
16	105	3	2.46	(1.91–3.11)	68	1	3.23	
17	120	4	2.38	(2.09–3.02)	72	1	3.90	
18	150	2	3.99	(3.51–4.47)	84	1	4.47	
19	170	4	4.12	(3.16–5.48)				
19–20	185	2	4.35	(2.41–6.29)				
20	218	2	9.16	(8.52–9.79)				

constant level of DNA per embryo. Subsequent development is accompanied by a net synthesis of DNA. These results are not in complete agreement with the observations of Hoff-Jorgensen ('54); Gregg and Lovtrup ('55) and Lovtrup ('55) who demonstrated a relatively constant amount of DNA throughout segmentation until a late blastula stage.

The average of 7 determinations at 15° yields a value of 0.023 micrograms of DNA in the fertilized egg. Gregg and Lovtrup report a value of 0.065 μg for the same species. However, if the content of free deoxyribosides is calculated as DNA, the total amount would be 0.052 μg, which is in good agreement. Just as was found by other investigators, here too, the content of DNA in the fertilized egg is far in excess of that to be expected for a diploid nucleus. The determinations of Sze ('53) indicate that in *Rana pipiens* a diploid adult nucleus contains 1.04×10^{-5} micrograms of DNA. The excess DNA corresponds, therefore, to approximately 2,200 diploid nuclei which is equivalent to an early blastula. Since cells are dividing quite rapidly during early cleavage, one may assume that a major portion of the nuclei are in the process of duplication, and would be close to the tetraploid rather than diploid condition. If this is so, then the amount of DNA in the fertilized egg could account for approximately 1,100 tetraploid nuclei. This corresponds to a very early blastula of approximately 18 hours at 15°C.

The pattern of synthesis of DNA is suggestive of exponential growth. If these values are plotted semi-logarithmically, as shown in figure 1, two straight lines may be fitted to the data. The break in the curve occurs at the gastrula stage (S11) at both temperatures and indicates that there is a decrease in the rate of synthesis after gastrulation. These curves are not in agreement with that reported by Sze ('53) who suggested that DNA was synthesized at a constant rate throughout development, at 15°C, with a resulting single straight line in a semi-log plot.

However, the curves shown in figure 1 do exhibit remarkable similarity with a logarithmic plot of the increase in cell

number during development, as shown in figure 4 of Sze's paper ('53). The curve for cell number is also characterized by an inflection during gastrulation, indicating a reduction in the rate of cell division after gastrulation. According to

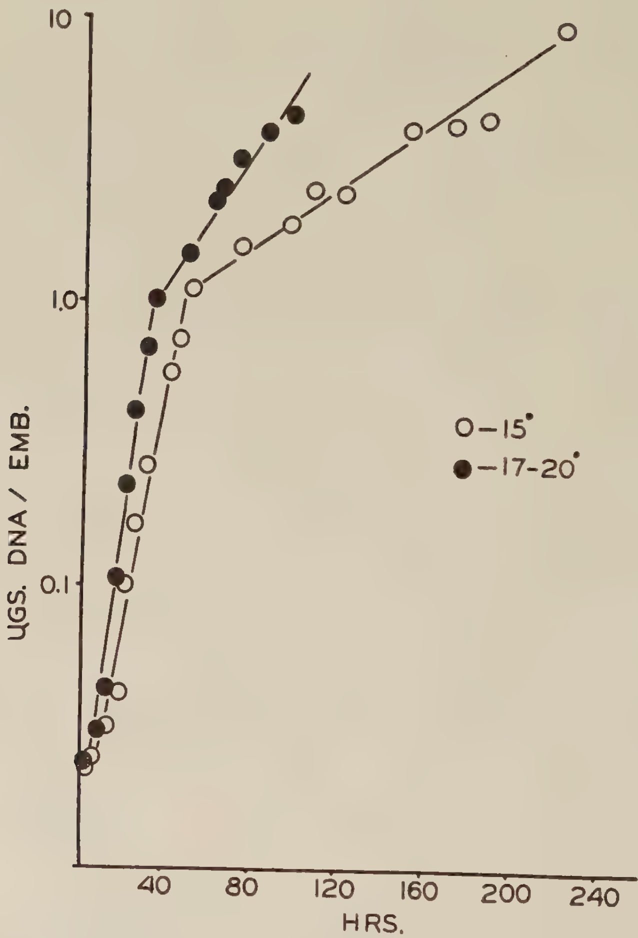

Fig. 1 A semi-logarithmic plot of the synthesis of DNA during development.

Sze, the rate of cell multiplication in the first day after fertilization is 26 times that after the beginning of gastrulation.

If the synthesis of DNA is correlated with the increase in cell number, then it should be possible to relate the rate of

synthesis of DNA with the rate of cell multiplication. Using Sze's equations for the calculation of cell number at specific stages, it is possible to relate the number of cells at a particular stage with the amount of DNA found at that stage. If plotted for development on a log-log plot, it should be possible to determine whether there is any linear relationship between these two processes. The curve in figure 2 does exhibit a

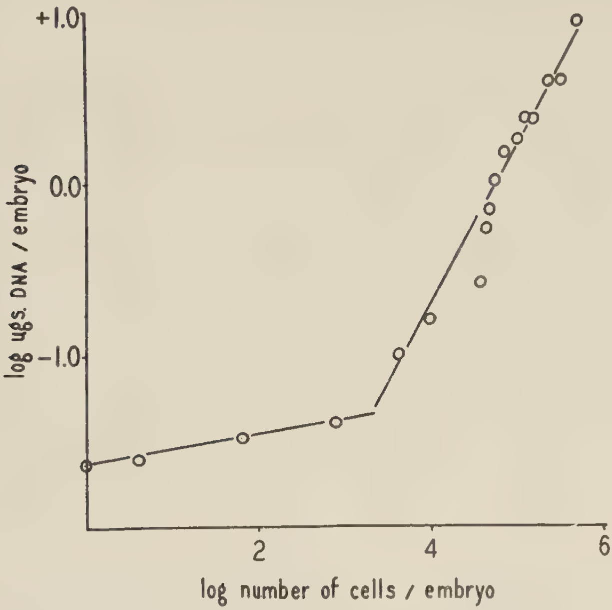

Fig. 2 The relationship between the number of cells per embryo and the content of DNA at the same stage. Temp. 15°C.

heterauxetic growth pattern and suggests that the rate of DNA synthesis is dependent on the rate of cell multiplication. During the first 16 hours, the rate of DNA synthesis is less than the rate of cell division. This is shown in the first portion of the curve. However, at stage 8^{-}–8, an early blastula of approximately 1,000 cells, the rate of DNA synthesis becomes equal to the rate of cell division, since the slope of the curve is approximately one.

During the first 12 hours of development, it can be shown that the amount of DNA per embryo is far in excess of the amount that would be expected, assuming the diploid value for DNA per nucleus. At the point of inflection of the curve, the theoretical amount of DNA per embryo as calculated (using value of 1.04×10^{-5} μg DNA per diploid nucleus (Sze, '53) and equations for determining number of cells at a particular stage) corresponds to the amount per embryo assayed. This is the situation which continues through subsequent development.

One may inquire as to whether the acid soluble, "free" deoxyribosides serve as precursors for the synthesis of DNA during cleavage. In table 3, the relationship between acid soluble deoxyribosides and the synthesis of DNA is shown. The fertilized egg contains 0.024 μg of free deoxyriboside, which is 57% of the total available deoxyriboside activity. This amount of deoxyriboside, if available as a precursor for DNA synthesis, is capable of providing for the synthesis of approximately 1,800 diploid cells, or an early blastula.

During the first 12 hours of cleavage, these deoxyribosides represent approximately 70% of all the available deoxyriboside activity. This period is also characterized by a total net synthesis of 0.055 μg/embryo of deoxyribosides; 0.013 μg synthesized as DNA and the remainder as acid soluble deoxyriboside. Thus, there seems to be no apparent precursor relation during very early cleavage. However, in the subsequent period (from 12–20 hours), a decrease in free deoxyriboside is accompanied by rapid DNA synthesis. It can be shown that approximately 40% of the newly synthesized DNA could have been derived from utilization of previously synthseized deoxyribosides.

These relationships are best shown in figure 3 which is a plot of the ratio of micrograms of acid soluble deoxyriboside activity at a particular stage to the micrograms of DNA at the same stage. It clearly indicates the apparent intense synthesis of free deoxyribosides during early cleavage and their rapid utilization thereafter. It indicates that during segmen-

TABLE 3

The relationship between acid-soluble deoxyribonucleoside activity and the synthesis of DNA during development

HOURS	STAGE	μG DNA/EMB.	μG DNA CALC. AS DR	μG DR/EMB.	TOTAL DR ACTIVITY μG/EMB.	% AS DR	TOTAL DR ACT. CALC. AS DNA μG/EMB.
2	2	0.024	0.018	0.024	0.042	57.1	0.057
5–8	6	0.031	0.023	0.053	0.076	69.7	0.103
9–12	8⁻	0.042	0.031	0.066	0.097	58.0	0.132
13–17	8⁻–8	0.108	0.080	0.063	0.143	44.0	0.195
18–20	8	0.228	0.168	0.015	0.183	8.1	0.249
21–25	9	0.408	0.300	0.019	0.319	6.0	0.434
26–31	10	0.689	0.505	0.015	0.520	2.9	0.706
32–36	11	1.00	0.735	0.016	0.751	2.1	1.02
40–48	12⁺	1.47	1.08	0.016	1.10	1.5	1.49

DR represents the acid soluble "free" deoxyribonucleoside activity. Temp. 17–20°C. Total DR Activity is a summation of columns 4 and 5.

tation, up to the formation of the mid blastula (stage 8), there is a high ratio of acid soluble precursors to DNA. However, in later development, as the rate of cell multiplication diminishes, the ratio of deoxyriboside precursors to DNA decreases to extremely low levels.

It may be noted from the last column of table 3 that the total deoxyribonucleosides per embryo, when calculated as DNA, indicates a net synthesis of DNA throughout development. This is to be compared to the results of Hoff-Jorgen-

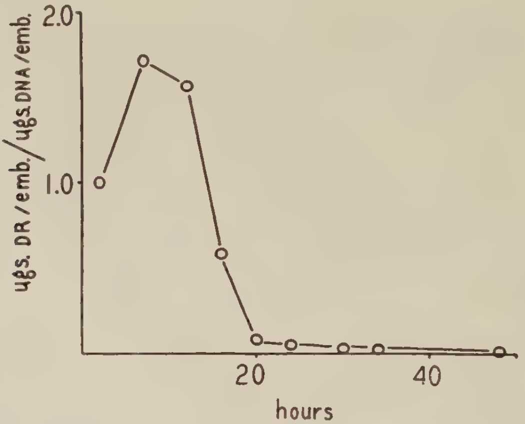

Fig. 3 Ratio of acid soluble deoxyribosides per embryo to the content of DNA during early development. Temp. 17–20°C.

sen ('54) who, analyzing total deoxyriboside activity, finds no net synthesis during cleavage.

DISCUSSION

It can readily be seen that the method of extraction influences the pattern of DNA synthesis obtained. Curiously, all methods utilizing a preliminary acid treatment (1% perchloric or 10% trichloracetic acid in the cold) provide evidence for a distinct net synthesis of DNA during early cleavage (Brachet, '54; Sze, '53; Kutsky, '50). Moreover, acid extraction demonstrates the existence of a relatively large precursor pool of soluble deoxyribosides and/or deoxyribotides, which,

during early cleavage, represents 57–70% of the total available deoxyriboside activity. On the other hand, assay for DNA according to the procedure of Hoff-Jorgensen ('54), without acid treatment, consistently demonstrates the absence of net synthesis during early cleavage. Although there is no clear explanation for these differences, several considerations suggest themselves. One of these concerns the influence of accessory growth factors on *Thermobacterium acidophilus R 26* grown on Hoff-Jorgensen's basal medium (Siedler et al., '57). They found, using this basal medium, that accessory growth factors had a marked stimulatory effect in the presence of thymidine. Any such growth factors in embryo extracts would have less of an effect on the more complete medium used in these experiments. Since no independent check was made on the existence of these unknown factors in embryo extracts, it is impossible to evaluate their effect on these determinations.

This question of interference from growth factors introduces some doubt as to the true nature of the acid-insoluble deoxyriboside activity. Its polynucleotide nature is based on the necessity for DNAase digestion to release all activity. Since it has been shown, however, that more than 50% of the "DNA" found by the Copenhagen group can be attributed to acid-soluble deoxyribosides, some caution is still necessary in interpreting the excess acid-insoluble, DNAase digestible material as "DNA." It is possible that it may be small polynucleotides or deoxyribonucleotides firmly bound to protein. Finally, it may yet be some non-specific growth factor interference. Obviously, considerably more experimental work is necessary before these difficulties are resolved.

The most interesting result derived from the present study is the demonstration that the synthesis of DNA parallels the pattern of cell multiplication. During cleavage, both the synthetic rate and the rate of cell division are rapid. Both exhibit a depression in rate during gastrulation and continue at a reduced rate during later development. Bragg ('38) has shown that the period of segmentation until the gastrula is

characterized by a high mitotic index. During gastrulation, the mitotic index diminishes to approximately one-tenth the previous level and continues at the reduced rate during neurulation. Thus, as a result of the application of an acid extraction technique to the microbiological assay procedure, it has been possible to demonstrate correspondence between the rate of cell division and the synthesis of DNA. The curve in figure 2 clearly demonstrates that after an early blastula stage, the rate of DNA synthesis and the rate of cell division are intimately related and approximately similar. Before this period, the rate of DNA synthesis seems to lag behind the rate of cell multiplication. This may have some implication as to the possible precursors utilized for the synthesis of DNA during this period. Solomon ('57) in studies on the chick embryo, has also shown the parallelism between mitotic index and the synthesis of DNA in early development.

The evidence strongly supports the hypothesis that deoxyribosides and/or deoxyribotides are serving as precursors for the synthesis of DNA during cleavage. Shortly after fertilization, a rapid synthesis of acid soluble deoxyribosides is evident at a time when the demands for DNA are limited (cell number increasing from 1 to 64 cells). Subsequent development to the late blastula imposes greater demands for new DNA since the number of cells produced from 12 to 20 hours is approximately 4,000; the DNA requirement is approximately 0.083 μg (assuming all nuclei during this period are tetraploid). During this latter phase there is a rapid depletion of acid soluble precursors such that 32–83% (depending on temperature) of this theoretical requirement could have been met by complete utilization of this precursor pool. The data indicate that 40% of the DNA synthesized could have been derived from acid soluble precursors.

In the last few years, numerous investigators have reported the existence of acid soluble deoxyribosides and deoxyribotides in egg cells, mammalian tissues and tumors. (Levenbook et al., '53; Marshak and Marshak, '54, '55; Schneider, '54, '55, '57a and b; Lansford and Shive, '55). Schneider has

suggested that these compounds serve as precursors for DNA synthesis since he finds in regenerating liver that an increase in these compounds occurs prior to an increase in cell division. Lansford and Shive report a higher ratio of free thymidine to DNA in mitotically active tissues than in non-dividing tissues, which agrees with the observations in figure 3 where segmentation is characterized by a much higher ratio than is the period following gastrulation. Finally, Travaglini and Schultz (Schultz, '56) have shown a correlation between deoxyribosides and DNA synthesis during 20 hours development of the *Drosophila* egg.

After gastrulation, the proportion of cells participating in cell division falls off considerably and the majority of cells undergo progressive differentiation. Centers of cell division are set up which results in a differential distribution of the mitotic rate (Bragg, '38). The synthesis of DNA is now confined to well-defined areas of the embryo and proceeds at a rate characteristic of the morphogenetic events unfolding at these regions. The rate of synthesis diminishes to what may be taken as indicative of a steady state during neurulation since the mitotic index for the whole embryo from mid gastrula to late neurula remains constant. Is it possible that the relatively constant levels of acid soluble deoxyribosides characteristic of gastrulation and neurulation reflect this equilibrium situation? It seems likely that the acid soluble deoxyribosides during these stages function as a labile precursor pool providing for the synthesis of DNA in those localized centers of the embryo in which cell division continues.

At tailbud (stage 17), there is a gradual increase in the levels of free deoxyribosides. According to Lovtrup ('55), the synthesis of DNA from stage 18 to 21 stops in *Rana platyrrhina*. This is followed by a rapid synthesis in later larval stages which is assumed to be associated with the differentiating endoderm. Although there is no evidence of a cessation of DNA synthesis in *Rana pipiens* at these stages, the behavior of the free deoxyribonucleosides after stage 17 suggests that a store of DNA precursors is accumulating in preparation

for some later period of rapid DNA synthesis, possibly in a manner suggested by Lovtrup.

To return to the original question as to whether "partial" or "total" synthesis is the essential mechanism for the synthesis of DNA during early amphibian development, we have evidence that clearly indicates that the patterns of synthesis are considerably more complicated than the terms "partial" or "total" would imply. Evidently, during early cleavage, there may be at least two sources for nuclear DNA; first, a pool of acid soluble deoxyriboside precursors previously synthesized in the oocyte and secondly, a *de novo* synthesis of these precursors during the first 12 hours of development which is capable of providing for almost 40% of newly synthesized DNA in subsequent development to the blastula stage. The remaining 60% may be derived, in part, from the excess "cytoplasmic DNA" which is exhausted at 20 hours of development; i.e, the amount of DNA per embryo at that stage is equivalent to the theoretical expectations based on cell number. This corresponds to the point of inflection in figure 2 and offers a possible explanation for the observation of a lack of correspondence between the rate of DNA synthesis and cell multiplication during this early period. Bieber et al. ('54) have evidence which suggests that the course of amphibian development is characterized by three period specific patterns of DNA synthesis; the period up to the late gastrula (stage 12) characterized by a utilization of preformed nucleic acid (excess DNA); the period from stage 12 to stage 15 characterized by the utilization of smaller nucleic acid precursors (purines) and the period after stage 15 characterized by *de novo* synthesis from small molecules. These conclusions are based on deoxyribose and UV analyses of hot and cold TCA extracts of embryos. It is possible that these analyses are complicated by the presence of large amounts of RNA and free purines found in the embryo. Moreover, it is quite possible that the pathways mentioned above are not necessarily the exclusive pathways for DNA synthesis during the specified period. Several mechanisms may be operating simultaneously. Isotope incorporation

studies in mammalian tissues (Brown, '56) have led to the hypothesis that at least two pathways may be functioning for the synthesis of DNA in mitotically active tissues; *de novo* synthesis from small molecules and transpurination or purine exchange.

After the exhaustion of the "DNA" reserve and of the deoxyriboside pool at mid blastula (stage 8), later development seems to be characterized by a "total" or *"de novo"* pathway for DNA synthesis, although, here too, the situation is probably more complicated. There may be other mechanisms for the synthesis of DNA which are, as yet, incompletely defined. The possibility that RNA may make a contribution to the pool of DNA precursors is certainly to be entertained since recent isotope studies indicate a utilization of intact ribonucleosides for the synthesis of DNA in mammalian tissues (Rose and Schweigert, '53; Roll et al., '56). The fact that the RNA: DNA ratio may be as high as 200 (Lovtrup, '55) and the lack of any net synthesis of RNA during development to the neurula suggests the possibility of RNA serving as an indirect precursor for DNA. Finally, Steinert ('51) has shown that the levels of free purines diminish rapidly after gastrulation suggesting their utilization in the synthesis of nucleic acids. He has later confirmed this observation by demonstrating the incorporation of labelled adenine and hypoxanthine into nucleic acids after injection into the gastrula ('55). These observations seem to support the suggestion by Bieber that the period from stage 12 to stage 15 is characterized by the utilization of small nucleic acid precursors such as purines. It is obvious that more additional work along these lines is required before any understanding of the complex biochemical patterns of nucleic acid synthesis during development is possible.

SUMMARY

The pattern of synthesis of DNA and the levels of acid soluble "free" deoxyribonucleosides and deoxyribonucleotides have been determined for early embryonic development of *Rana pipiens*. By the use of a modification of the micro-

biological assay procedure of Hoff-Jorgensen for the determination of deoxyriboside activity, it has been possible to demonstrate that the fertilized egg contains 0.023 μg of DNA which is in excess of that to be expected assuming the equivalence of egg and sperm nuclei. Moreover, it has been possible to demonstrate a net synthesis of DNA during early cleavage which parallels the rate of cell division. The rate is most rapid during cleavage when mitotic activity is the highest and falls off during gastrulation and neurulation when the increase in cell number also diminishes. For both these periods the respective rates are constant.

The levels of acid soluble deoxyribonucleoside activity in early cleavage suggest that these may serve as precursors for the synthesis of DNA; they may contribute to the formation of 40% of the new DNA synthesized.

The implications of these findings are discussed in relation to the general problem of total or partial synthesis of DNA during early amphibian development.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Jack Schultz for his excellent criticism and helpful advice during the course of this investigation. I wish, also, to express my thanks to Miss Elizabeth Travaglini for her gracious encouragement in developing the assay methods and to Miss Carole Bradt for carrying out the assays.

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THE INCORPORATION OF P^{32} AND GLYCINE-2-C^{14} INTO NUCLEIC ACIDS DURING EARLY EMBRYONIC DEVELOPMENT OF RANA PIPIENS [1]

PHILIP GRANT [2]
Institute for Cancer Research,
Philadelphia, Pennsylvania

ONE FIGURE

INTRODUCTION

In another investigation (Grant, '58), evidence was obtained which demonstrated a net synthesis of DNA during early cleavage stages in *Rana pipiens* development. Moreover, a very early synthesis of acid soluble deoxyriboside precursors was demonstrated, which suggested that these substances may serve as intermediates in the synthesis of DNA during the latter phases of segmentation. Contrary to the observations of Hoff-Jorgensen ('54) and Lovtrup ('55) which suggest a preferential utilization of cytoplasmic polynucleotide DNA precursors during cleavage, the results cited above suggest that several mechanisms for the synthesis of DNA may operate during this period. One of these may involve a *de novo* synthesis from small molecules.

That a *de novo* synthesis may occur during early cleavage is suggested by the results of Flickinger ('54) who found that labeled carbon dioxide is incorporated into DNA during 5 hours exposure of the fertilized egg. The levels of incorporation were considerably lower than those observed in later

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[2] Present address: Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

stages. Bieber et al. ('55), however, studying the incorporation of formate C^{14}, found little incorporation into total nucleic acids prior to gastrulation. Incorporation was most evident in the late neurula and they suggest that this supports the view that *de novo* synthesis of nucleic acids occurs some time after the onset of neurulation.

The experiments to be presented in this study represent a preliminary attempt to determine whether *de novo* synthesis of DNA occurs during cleavage. They were also designed to learn whether *de novo* synthesis becomes a more important pathway for DNA synthesis after gastrulation. An approach to this question would be a comparison of the incorporation of several *de novo* precursors (P^{32} and glycine-2-C^{14}) at different stages of development with the incorporation of precursors involved in "partial" synthesis of DNA (thymidine, etc). Only the preliminary experiments with phosphorus and glycine are to be reported at this time.

MATERIALS AND METHODS

A. P^{32} incorporation. For the phosphorus experiments, a modification of the procedure employed by Kutsky ('50) was used. Females were injected with 1 ml of Ringer's solution containing $NaHP^{32}O_4$. This was followed by an injection of pituitary glands to induce ovulation. The frogs were kept at 15°C for 24–48 hours at which time the eggs were stripped and fertilized. They were allowed to develop at (20–22°C) in finger bowls. At appropriate intervals, 25–50 embryos in duplicate samples were taken for analysis.

The embryos were fractionated according to a modified Schmidt-Thannhauser procedure ('45). The acid-soluble fraction, RNA, DNA, lipid and protein were collected and analyzed for activity and for phosphorus. The RNA was obtained from the alkali digest after precipitation of the "phosphoprotein phosphorus" with magnesia mixture. Thus, the extent of contamination of the RNA fraction was minimized. All fractions were hydrolyzed in 60% perchloric acid, the inorganic P precipitated with magnesia mixture and the

precipitate collected on a filter paper disc for counting. After activity had been determined and corrected for decay, the filter paper was eluted in 1 N sulphuric acid and the phosphorus determined according to the procedure of Berenblum and Chain as modified by Wiame ('49).

Results for phosphorus are expressed as relative specific activity which is calculated as cts/min/µg P divided by total activity per embryo times 100.

B. Glycine-2-C^{14} incorporation. To obviate the difficulties associated with glycine immersion experiments (Friedberg and Eakin, '49), glycine-2-C^{14} was injected directly into the fertilized egg in a Niu-Twitty carrier solution (Flickinger, '49). Although injections of isotopes into amphibian embryos have been described before (Steinert, '55) this is the first time that injections into fertilized eggs have been used in this manner. Steinert injected into the blastocoele of the blastula or gastrula. Injections have two distinct advantages over immersion experiments: (1) the amount of label per egg is known and (2) factors of diffusion and permeability do not influence the incorporation patterns. One, thus, takes advantage of the cleidoic nature of the amphibian egg. Introduction of a known amount of label permits an analysis of its distribution with time. This, obviously, is quite different from the P^{32} situation in which the mature egg has been exposed to the label for 24 hours while in the ovary. This permits organic combination of the phosphorus whereas glycine injections simply contribute labeled glycine to the free glycine pool of the egg.

Injections were accomplished using an Emerson Micromanipulator. Micropipettes of 10–25 mµ diameter at the tip were employed and injections were either in the pigmented animal half of the egg or the yolk filled vegetal region. In the case of later stages, injections were both dorsal and ventral. Injections of the Niu-Twitty carrier solution resulted in 70–80% normal development while injection of glycine-2-C^{14} (21 mg/ml, 1.1 mc/millimole) resulted in 50–70% normal development. Only normal embryos were used for analysis.

Approximately 4.0×10^{-3} ml of solution was injected into each egg or embryo. This was determined by calculating the volume of the pipette using an ocular micrometer and reading the volume delivered directly. Experiments with either P^{32} or glycine-2-C^{14} wherein a series of eggs were injected with the same volume of solution, washed several times, dried on a planchet and counted, yielded a 5–10% error in injection.

Two types of experiments were performed: (1) injection of the fertilized egg and the Shumway stage 11 gastrula with equivalent amounts of labeled glycine and following development for 80–90 hours, taking samples for analysis at appropriate intervals and (2) injection of 5 to 6 different stages from the same batch of eggs with equivalent amounts of radioactive glycine and following incorporation over a 6 hour period, taking samples at 2, 4 and 6 hours. In these experiments, dorsal and ventral injections were compared.

In all experiments, 20 eggs (injected with labeled glycine) or embryos were placed in a 15 ml Warburg vessel containing (20%) KOH-soaked filter paper in the center well. They were incubated at 20°C with shaking and samples were taken at frequent intervals for analysis. The embryos were removed, washed several times in 10% Holtfreter's solution and extracted immediately in ice cold 10% TCA. They were carried through the Schneider modification of the Schmidt-Thannhauser procedure ('46). RNA was separated from protein and DNA by alkaline hydrolysis and acid-alcohol precipitation of the DNA-protein. DNA was separated from protein by hot 5% TCA. The following crude extracts were collected and analyzed for activity only: acid soluble, RNA, DNA, lipid, protein, CO_2 and the incubation medium itself. Aliquots of each fraction were placed in an aluminum planchet (at minimum thickness) and counted in a Nuclear Chicago Automatic sample changer with an end window tube. A minimum of 1000 counts was counted resulting in a counting error of 3–5%. No corrections for coincidence or absorption were necessary.

Values were calculated for total activity in each fraction by making the necessary volume corrections and the per cent of total activity recovered per egg was then determined. Data are expressed as per cent incorporation. Total recovery varied for each experiment and was difficult to determine accurately since loss to the medium varied with different stages and in some cases, where abnormal embryos were found, the loss to the medium was even greater. Roughly estimated, recovery varied between 60–80%.

One experiment of the injection type was also completed for P^{32}.

RESULTS

A. Phosphorus incorporation. The changes in the relative specific activity during development for the acid soluble phosphorus, RNA phosphorus and DNA phosphorus are shown in figure 1. This curve represents the most complete of three experiments. The other experiments were essentially identical. Values for lipid phosphorus and protein phosphorus are not shown since these fractions did not exhibit significant changes during the period under study. For the former, the relative specific activity increased slightly from 0.87 at stage 2 to approximately 3.0 at the end of neurulation. There was no change in the specific activity of the protein P. It remained within the range 0.19–0.22.

During the period from fertilization to the mid gastrula, the acid soluble fraction is essentially constant. On gastrulation and through neurulation there is a rapid decrease in specific activity. Since the acid soluble phosphorus per embryo is constant throughout this period (Kutsky, '50), the decrease represents rapid turnover of the acid soluble pool with a consequent funnelling of labeled precursors into the acid insoluble fractions. These precursors are evidently entering metabolic pathways associated with the synthesis of nucleic acids, since these two phosphorus fractions exhibit the greatest increases in specific activity. The RNA P exhibits only a gradual increase during cleavage and gastrula-

tion with a slightly greater rise during neurulation. The level increases gradually during later development.

The "DNA" P exhibits a surprising pattern of incorporation. There is no evidence of phosphate uptake during the first 12 hours of development (stage 7–8⁻). Subsequently, there is a rapid linear rate of incorporation through gastrulation and neurulation which is considerably greater than

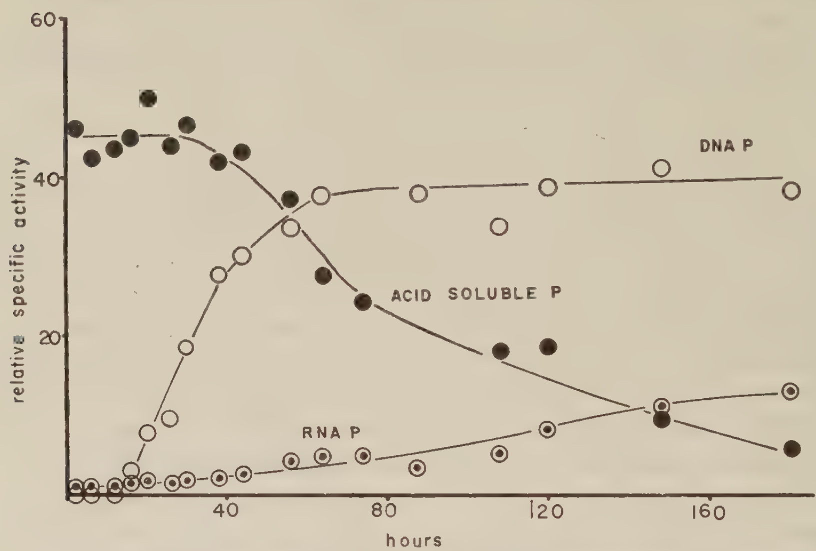

Fig. 1 Relative specific activity of phosphorus fractions during development. Temp. 18–20°C. Relative specific activity equals $\frac{\text{cts/min/}\mu\text{g P}}{\text{total cts/min/emb.}} \times 100$.

the RNA P. From 16 hours (stage 8), the ratio of RNA : DNA specific activities varies from 1.0 to 0.16 at stage 16. The period after neurulation is characterized by a constant specific activity.

The data for phosphorus incorporation has been calculated on the basis of per cent incorporation and is shown in table 1. This has been done so as to make the data comparable to the glycine incorporation patterns. It is interesting to note that as a result of these calculations, the incorporation into DNA and RNA is approximately identical; the RNA being only

TABLE 1

Per cent incorporation of P^{32} during development

STAGE	NO. EXP.	HOURS	PHOSPHORUS FRACTIONS				
			ASP	LP	RNAP	DNAP	Prot. P
1	1	0–1	94.3	1.7	1.2	0.0	2.9
2	3	2	93.5	3.0	0.73	0.0	2.7
6–7	2	7	93.4	3.2	0.85	0.0	2.5
8	2	16	91.1	4.8	1.1	0.13	3.0
8+	1	19	89.9	6.2	1.3	0.37	2.3
9	3	21	90.5	5.1	1.3	0.66	2.5
10	3	26	89.5	5.6	1.6	0.94	2.4
10+	1	30	86.7	5.6	2.0	3.4	2.4
11	2	34	87.2	5.9	1.7	2.4	2.9
11+	1	38	82.5	6.0	3.5	5.6	2.5
12	3	42	81.1	7.6	3.2	5.8	2.5
12+	1	46	79.4	9.3	—	8.2	2.6
13	3	52	74.9	7.7	4.7	10.1	2.6
13+	1	58	70.1	7.3	6.7	12.8	3.1
14	2	65	63.1	9.3	8.6	15.9	3.2
17	1	84	55.3	9.9	12.7	19.3	2.8
17+	1	90	47.6	10.7	15.8	22.3	3.4
18	1	110	32.7	11.2	24.4	28.2	3.4
20	1	150	21.8	12.7	30.5	30.9	3.9

ASP, acid soluble phosphorus; LP, lipid phosphorus; RNAP, ribonucleic acid phosphorus; DNAP, deoxyribonucleic acid phosphorus; Prot. P, protein phosphorus. (% of total activity recovered.)

slightly lower. They both reach approximately 30% incorporation in 120 hours. This data is in excellent agreement with the observations of Kutsky ('50). In terms of distribution of phosphorus, RNA and DNA take up almost equal proportions; however, as has been seen, on a specific activity basis, the behavior of these fractions is distinctly different.

An experiment, in which labeled inorganic phosphate was injected into the fertilized egg with subsequent development and analysis at different stages (as in glycine experiments) gave almost identical results as those shown in table 1, except for one very significant difference. Under the conditions of direct injection into the free inorganic phosphate pool, there was a measurable incorporation into DNA phosphorus during early cleavage.

Obviously the "DNA" fraction is crude. The extent of contamination by RNA is unknown. However, the fact that no incorporation was found in the DNA during the first 12 hours, indicating no contamination of this fraction, does suggest that the levels for "DNA" may be significant, albeit somewhat lower than observed.

B. Incorporation of glycine-2-C^{14}. A balance sheet of glycine metabolism was obtained after injection of radioactive glycine into the fertilized egg and gastrula respectively. Considering the marked differences between the two stages, particularly with reference to cell number, morphogenetic movement and differentiation, it seemed likely that the overall pattern of glycine metabolism would reflect these differences.

In tables 2 and 3, the pattern of incorporation during development is shown for the fertilized egg and the gastrula. It is clear that the metabolism of glycine is approximately twice as rapid after gastrulation than it is after fertilization. The acid soluble fraction attains equilibrium 60 hours after fertilization whereas it equilibrates in 30 hours after gastrulation. All fractions exhibit the heightened incorporation after gastrulation, except the lipid which is essentially the same.

TABLE 2

The per cent incorporation of glycine-2-C^{14} during development after injection into the fertilized egg

STAGE	HOURS	FRACTIONS					
		AS	L	RNA	DNA	P	CO_2
7 (3)	8	91.8	2.1	3.8	0.26	1.5	0.52
8 (1)	17	86.1	2.6	6.6	0.36	3.5	0.84
9 (3)	21	79.0	8.8	7.5	1.1	2.2	1.3
11 (3)	34	56.6	17.2	14.1	3.4	3.8	4.9
12 (1)	42	52.4	8.1	18.1	5.4	4.8	11.4
13 (2)	50	25.2	20.8	27.7	11.4	6.7	8.0
14 (1)	62	14.3	14.0	38.1	11.9	4.4	17.9
17 (1)	78	13.6	23.4	23.5	10.6	3.8	25.2

AS, acid soluble; L, lipid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; P, protein; CO_2, carbon dioxide. Per cent incorporation measured as per cent of total recovered activity. Temperature of incubation, 20°C. Nos. in parentheses equal no. of experiments. Injection into vegetal region.

TABLE 3

The per cent incorporation of glycine-2-C^{14} during development after injection into the gastrula

STAGE	HOURS	FRACTIONS					
		AS	L	RNA	DNA	P	CO_2
12 (2)	8	80.0	2.2	11.7	2.2	2.3	1.4
14 (1)	26	17.1	7.0	45.2	9.0	7.1	14.5
18 (2)	45	11.1	19.2	43.8	10.6	6.7	20.1
20 (2)	66	9.0	19.4	28.1	10.9	6.1	26.3
22 (1)	93	4.7	4.1	19.3	8.2	0.6	63.2

Per cent incorporation measured as per cent of total recovered activity. Temperature of incubation 20°C. Symbols same as in table 2. Injection into vegetal region.

As one would expect, the behavior of the "RNA" and "DNA" fractions is related to the acid-soluble pattern. Both nucleic acids attain maximum incorporation at the point when the acid-soluble precursor pool has equilibrated. This is true for the gastrula as well as the fertilized egg. Moreover, the maximum incorporation attained by the "DNA" is identical under both situations; approximately 10–11%. As for the RNA, a greater incorporation (45%) is reached after injection into the gastrula, although this may not be significant. Finally, the stages at which these maxima are reached are similar in both instances; namely, stage 14.

It is interesting to note that the incorporation into DNA remains constant (at 11% in both cases) after the point of maximum incorporation, in spite of a depleted acid soluble pool. This suggests maximum retention of the label, supporting the concept of relative metabolic stability of the DNA. The RNA, however, exhibits a decline in per cent incorporation, suggesting rapid turnover and replacement from a relatively "cold" acid soluble precursor pool.

Comparison of the glycine incorporation data with the per cent incorporation of phosphorus indicates that for the DNA, the patterns are essentially similar, except for the crucial first 7–8 hours. By 50 hours (stage 13) both have been incorporated to the extent of 10 to 11%. The glycine levels are then constant since the acid soluble glycine pool has equilibrated at low levels (approximately 14%) whereas the phosphorus incorporation continues to increase since the phosphorus precursor pool is still at high levels of activity (75%). The RNA, however, differs in its pattern of incorporation with glycine, exhibiting a more rapid rate.

Clearly, the incorporation into RNA is considerably more rapid than into DNA on a per cent incorporation basis. Calculated as specific activity, however, this presumably would not be the case, in view of the greater absolute amounts of RNA per embryo. During cleavage, the RNA may incorporate as much as 20 times the amount of glycine as DNA, however, the ratio of the absolute amounts of RNA:DNA per embryo in these stages may be as high as 200 (Lovtrup, '55).

There seems to be a significant level of incorporation into the DNA during early cleavage. The levels are low (0.2–0.4%) and possibly may be attributed to contamination by protein and/or RNA. Le Page and Heidelberger ('51) using a similar extraction procedure for mammalian tissues report 5–8% contamination by protein. In one experiment, using their modification of the procedure so as to minimize this contamination, incorporation can be shown to occur up to 0.1%, 6 hours after injection into the fertilized egg. Some question may still exist, however, as to the significance of these low levels of incorporation during cleavage.

C. A comparison of initial rates of glycine-2-C^{14} incorporation in different developmental stages. The patterns of incorporation are complicated by the rapidity with which glycine participates in numerous metabolic pathways (Weinhouse, '55) so that long term experiments may not reflect initial patterns of glycine incorporation.

In the 6-hour experiments, it became obvious that the site of injection influenced the pattern of incorporation. Injection into the dorsal region of the embryo (or animal half in earlier stages) demonstrates a more rapid utilization of glycine than similar injections into the ventral or yolk filled regions of the embryo. This is shown in table 4. For identical stages, the acid-soluble incorporation decreases more rapidly over the 6-hour period in a dorsally injected embryo. Similarly, incorporation into RNA and DNA is greater. This differential activity suggests that the injected glycine does not diffuse rapidly throughout the embryo; rather, it tends to remain localized in the area of injection and is rapidly incorporated into the surrounding cells. The results of Flickinger ('54) lend additional support to the conclusion that this differential response may be a significant phenomenon. He finds that the dorsal ectoderm of a gastrula has higher specific activities than the ventral, yolk laden cells.

It can be seen from table 4 that, irrespective of the site of injection, later stages, apparently, exhibit a more rapid

TABLE 4

Per cent incorporation of glycine-2-C^{14} into nucleic acids after injection at different stages; six-hour incorporation

STAGE	HOURS	AS		RNA		DNA	
		D	V	D	V	D	V
2 (3)	2	87.7	97.0	12.0	2.0	0.52	0.13
	4	80.6	97.5	18.4	2.5	1.1	0.15
	6	64.6	92.9	32.7	7.0	1.6	0.25
8^- (2)	2	92.4	98.1	7.3	1.8	0.22	0.07
	4	83.8	95.4	15.2	4.4	0.94	0.34
	6	73.5	93.4	24.2	6.2	2.3	0.36
8^+ (1)	2	80.2		18.5		1.3	
	4	44.8		48.9		4.6	
	6	54.7		40.8		4.5	
10 (1)	2		96.9		2.8		0.40
	4		93.3		6.2		0.44
	6		91.5		7.5		1.1
11^- (2)	2	64.4		33.8		1.7	
	4	48.4		48.8		3.0	
	6	37.0		58.8		4.2	
12 (1)	2		93.7		6.0		0.43
	4		86.3		12.8		0.82
	6		78.4		19.9		1.6
14 (2)	2	51.9	93.9	46.5	5.9	1.5	0.34
	4	29.6	85.2	67.6	13.6	2.8	1.1
	6	24.8	77.9	72.2	20.0	3.1	1.8

Activity expressed as per cent of total recovered. D is dorsal injection; V is ventral injection. Nos. in parentheses equal no. of experiments.

utilization of the injected glycine. The acid soluble fraction exhibits progressively increasing rates of depletion which, in turn, implies a greater incorporation into nucleic acids. Moreover, the stages may be grouped into classes based on their respective patterns. Stages 2 and 8^- are very similar, both exhibiting the least glycine utilization. Stages 8^+ and 10 and 11^- exhibit intermediary patterns, whereas stages 12 and 14 are the most active and are essentially alike. This would tend to suggest that some time during gastrulation there may be an "activation" or stimulation of certain pathways leading to the synthesis of nucleic acid.

DISCUSSION

A comparison of the patterns of incorporation of phosphorus and glycine is complicated by several factors. The system itself is in a non-steady state, rapidly growing and differentiating biochemically as well as morphogenetically. Simple turnover analysis cannot apply. A most important difficulty arises when comparing the experimental designs for both isotopes. In the case of phosphorus, the isotope is incorporated under *in vivo* conditions while the egg is in the ovary. As for glycine, the endogenous glycine pool of the egg is considerably overloaded (approximately 50-fold) by the injection. This may alter the metabolism of glycine so as to force glycine into pathways which usually function at minimal levels. Under these conditions, it is possible that glycine will become incorporated into DNA. This has been shown to occur with phosphorus. Injection into the egg results in incorporation of P^{32} into DNA in early cleavage but not after injection into the female.

The pool sizes of phosphorus and glycine are probably quite different, varying with development. As one would expect, these acid soluble precursor pools equilibrate at different rates. Moreover, a major proportion of the glycine is eliminated as carbon dioxide. There is little or no phosphate loss. Finally, the DNA and RNA are both heterogeneous, being derived from different regions of the embryo characterized

by different rates of cell division in the case of the former and arising from cellular structures of variable turnover characteristics in the latter. This heterogeneity also varies with development.

Incorporation into DNA

The observations of phosphorus incorporation would tend to support the view that during the 12 hour period after fertilization, no *de novo* synthesis of DNA occurs. Although glycine incorporation seems to occur during early cleavage, the suggestions outlined in the previous discussion as to overloading the endogeneous glycine pool may explain these observations. Moreover, contamination may be more of a factor in these early stages. Thus, if we disregard the low levels of glycine incorporation during the first 8 hours of cleavage, the per cent incorporation patterns for glycine and phosphorus are quite similar. Both exhibit a marked increase in incorporation at approximately 17–20 hours and exhibit parallel incorporations through gastrulation (approximately 5–6%). They differ in later stages since equilibration of the respective acid soluble pools occurs at different rates.

This evidence seems to agree with previous observations concerning the utilization of two already existing precursor pools for DNA synthesis up to a mid blastula stage — free deoxyribonucleotides and deoxypolynucleotides (excess "DNA") (Grant, '58). The constancy of the acid soluble phosphate fraction during this period may indicate utilization of inorganic phosphate for the synthesis and accumulation of nucleic acid intermediates. Evidence for the synthesis of a large acid-soluble pool of deoxyribosides during the first 12 hours of development has been obtained (Grant, '58). Thereafter, the acid soluble precursors decrease as DNA is synthesized. This may correlate with the observed rapid linear increase in DNA specific activity (and per cent incorporation) concomitant with a decline in activity of the acid soluble phosphorus.

It should be emphasized, however, that in view of the difficulties of interpreting the data (outlined in the early part of the discussion), these conclusions are only tentative. The incorporation of glycine into DNA during early cleavage may be a significant pathway and the fact that it does not agree with the phosphorus observations could be explained by referring to differences in pool size, or to the relative ease of phosphate exchange and transfer.

The 6-hour glycine experiments illustrate two phenomena. Early cleavage stages are characterized by low levels of incorporation into DNA (stages 2 and 8^-). At the mid blastula stage (stage 8), a higher rate of glycine utilization is established which is essentially constant throughout gastrulation and neurulation (stages 11^- and 14). These suggestions of the establishment of a constant rate of glycine utilization during gastrulation and neurulation are in agreement with the observations on the behavior of free deoxyribosides during these periods. It was observed that this period is characterized by a constant level of deoxyriboside precursors (Grant, '58). Moreover, this constant pattern is established at a time when the rate of DNA synthesis and of cell multiplication diminish.

These results are not in agreement with those of Flickinger ('54) who found increasing specific activity of DNA with later stages of development. His experiments were of the immersion type, however, which provides for a continuous, high level external source of labeled substrate. The injection procedure introduces a limited amount of label which is subsequently diluted as synthesis takes place. Similarly, it is difficult to compare these observations to those of Bieber et al. ('55) on formate incorporation. They analyzed the hot TCA fractions of embryos immersed in formate-C^{14} so that it would be difficult to distinguish between RNA and DNA incorporation.

The short term experiments also illustrate the early establishment of biochemical heterogeneity in the embryo. The metabolism of glycine in dorsal regions of the embryo is considerably greater than in ventral regions.

Although it has been shown that both phosphate and glycine may be used for the synthesis of DNA, no clear relationship has emerged concerning stage-specific incorporation patterns. Although there is evidence for the *de novo* synthesis of DNA after the late blastula, it by no means implies that this is the only pathway for DNA synthesis during these periods. Steinert ('55) has demonstrated the utilization of adenine and hypoxanthine for the synthesis of nucleic acid purines after injection into the gastrula, suggesting the utilization of a pool of free purines found in the embryo during segmentation. Finally, the possible role of RNA metabolism in the synthesis of DNA is yet to be elucidated.

Incorporation into RNA

In view of the relatively constant amounts of RNA per embryo during early development (Lovtrup, '55; Kutsky, '50), incorporation of labeled precursors will be a measure of RNA turnover. The rapid incorporation of glycine into RNA after fertilization and during gastrulation is suggestive of rapid turnover. This is also indicated by the decline in per cent incorporation at a time when the acid soluble fraction has equilibrated. By contrast, the DNA exhibits no decline during this period.

The 6-hour experiments demonstrate an increase in glycine incorporation with later stages of development. High levels are attained during the neurula stages which correspond with the period at which a new synthesis of RNA occurs (Lovtrup, '55).

Is there any relationship between this active turnover and the synthesis of DNA? Observations of Rose and Schweigert ('53) and Roll et al. ('56) indicate the utilization of ribonucleosides and ribonucleotides for DNA synthesis in mammalian tissues. If, in the rapidly growing amphibian embryo, competition for a common pool of nucleotide precursors by separate synthetic pathways were to occur such that DNA synthesis was favored, some meaning may be derived from

the incorporation data. The rapid turnover of RNA would imply constant replacement from an acid soluble pool which is progressively diluted. However, the DNA, with its low metabolic turnover, would retain the label and exhibit progressively increasing specific activities until equilibrium is established. In later stages, as net synthesis of RNA occurs, incorporation into RNA is more efficient. The phosphorus specific activity curves illustrate this pattern quite well. At a time when DNA synthesis is intense, (during late blastula through early neurulation) incorporation of P^{32} is greater into DNA than into RNA. When RNA net synthesis is established during neurulation, there is increased incorporation into RNA with a concomitant leveling off of DNA incorporation. Considerably more experimental work is necessary to demonstrate the validity of this hypothesis.

SUMMARY

The patterns of incorporation of P^{32} and glycine-2-C^{14} into the nucleic acids of developing amphibian embryos were studied to determine the pathways of nucleic acid metabolism. Attention was directed to the *de novo* synthesis of DNA and a preliminary attempt was made to characterize periods of development with reference to patterns of nucleic acid synthesis. Two types of experimental situations were employed: One involving injection of the phosphate label into an adult female with subsequent induction of ovulation and analysis of developing embryos by means of a Schmidt-Thannhauser fractionation procedure. The other involved direct injection of the labeled precursor into the fertilized egg or later embryo followed by subsequent development and analysis.

The incorporation patterns of glycine and phosphorus into DNA were very similar except for the first 8 hours of cleavage. Under the conditions of direct injection into the egg, incorporation of glycine was observed, when no incorporation of phosphate occurred. Evidence was obtained which suggested that the first 12 hours of development are not characterized by a *de novo* synthesis of DNA. The incorporation

of glycine and phosphorus into DNA increases at mid blastula and continues at a constant rate during gastrulation and neurulation.

The metabolism of glycine after gastrulation is twice as rapid as after fertilization. Incorporation of glycine into RNA suggests an extremely high rate of turnover.

The metabolism of glycine in dorsal regions of the embryo is greater than in ventral regions.

The implications of these observations to the problem of nucleic acid synthesis during development are discussed.

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THE NUCLEUS IN RELATION TO PLASMAGEL STRUCTURE IN AMOEBA PROTEUS; A PRESSURE-TEMPERATURE ANALYSIS

HENRY I. HIRSHFIELD,[1] ARTHUR M. ZIMMERMAN AND DOUGLAS MARSLAND [2]

Washington Square College, New York University

ONE FIGURE

Interactions between nucleus and cytoplasm very obviously are important in the cell; and recently there have been quite a number of studies designed to analyze these inter-relationships. For example, Brachet ('54, '55) demonstrated nuclear dependence in regard to the synthesis of several cytoplasmic enzymes; D. Cohen ('56) showed that the DPN level in the cytoplasm of *Amoeba* falls off subsequent to enucleation; and Mazia and Hirshfield ('50) reported nuclear dependence with reference to the uptake of P_{32} by various cells. Holter and Kopac ('37), on the other hand, found that aminopeptidase activity in the *Amoeba* is more or less independent of the nucleus.

The purpose of the present study was to ascertain whether or not the nucleus may exert some influence on the formation and maintenance of plasmagel structure in *Amoeba proteus*. It seems well established (see Marsland, '56) that cytoplasmic gelation in cells generally represents an endothermic process — which might be nucleus-dependent for metabolic energy. Also it is known that the cycle of sol-gel changes, which is so intimately related to locomotion in Amoeba, can be modified

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greatly by changing the pressure and temperature conditions (Marsland, '56). Thus it seemed possible to test the question of nuclear dependence by studying nucleate and anucleate parts of the Amoeba under systematically varying conditions of pressure and temperature.

Amoeba proteus was found to be good material for the experiments. Quite quickly it can be cut, free hand, into roughly equal halves. Thus it is possible to obtain a considerable number of nucleate and anucleate halves within a reasonably short time (see below).

Previously it was shown (Marsland and Brown, '36; Landau, Zimmerman and Marsland, '54) that the plasmagel system of the intact *Amoeba* undergoes complete solation under suitably high pressure. In the pressurized specimens, the pseudopodia collapse and the cell rounds up into a motionless sphere. Moreover, it was shown that progressively higher pressures (3000–6000 lb/in.^{2}) are required to produce this reaction when the experimental temperature is increased, in the range between 10° and 25°C. In the present experiments, therefore, the rounding-up reaction has been taken as an index of gelational state. Specifically the nucleate and anucleated halves have been compared with each other and with whole *Amoebae,* with reference to relative susceptibility to high pressure solation, at three different temperatures (15°, 20° and 25°C).

METHODS

For each series of experiments about 100 *Amoeba,* washed twice in Brandwein solution, were transected into roughly equal halves — which then were separated into nucleate and anucleate groups (for technique, see Mazia and Hirshfield, '50). About 50 specimens from each group were used in any one experiment, which left a similar number from each group to serve as atmospheric controls. Also about 100 whole washed specimens were used in each series — 50 for each pressure-temperature treatment, and 50 as atmosphere controls.

Subsequent to cutting, all the specimens were placed in the temperature control housing (Marsland, '50) and equilibrated to the experimental temperature (15°, 20° or 25°C) for a minimum of 25 minutes. Then one selected group was placed in the microscope-pressure chamber (Marsland, '50) and subjected to an appropriate level of compression for 20 minutes. The first experiment in each series was started about one hour after the cutting operation began and the last of the series was completed within 2½ hours. In the several series, the order in which the groups were selected for experimental treatment was varied systematically.

At the end of the 20-minute compression period, before the pressure was released, counts were made as to how many of the specimens were completely rounded up and how many were not. Subsequent to compression, the specimens were saved for several days. Most of the specimens regained normal appearance and activity within some 10–20 minutes and the survival percentages in each of the treated groups were comparable to those of the corresponding untreated control groups.

RESULTS

The results of all the experiments are summarized in table 1 and plotted in figure 1. Here it may be seen that anucleate amoebae, compared to both kinds of nucleate specimens, displayed a distinctly greater susceptibility the pressure solation at each of the three different temperatures employed. In fact, judging on the basis of minimum pressure required to produce total rounding in approximately ⅔ of the specimens, the pressure differential at each temperature was of the order of 1500 lb/in.[2] Moreover, no plain difference could be found between whole amoebae and the nucleated half specimens.

DISCUSSION

The results indicate that the nucleus plays a very definite role in the maintenance of plasmagel structure in *Amoeba proteus*. Consistently the nucleated groups, both whole and

half specimens, displayed a greater resistance to pressure solation than did the anucleate groups. This implies (see Marsland, '50) that a more firmly set plasmagel structure is being maintained, at each different experimental temperature. Moreover, the results are in good agreement with the general observation that nucleate specimens maintain a higher level of amoeboid activity than do the specimens in the anucleate groups.

TABLE 1

TEMPERATURE	PRESSURE LB./IN.[2]	PERCENTAGE OF FULLY ROUNDED SPECIMENS[1]					
		NUCLEATE HALVES		ANUCLEATE HALVES		WHOLE AMOEBAE	
15°C	2000	2%	(48)	25%	(98)	——	
	2500	5%	(76)	67%	(83)	4%	(80)
	3000	22%	(102)	98%	(50)	16%	(200)
	4000	69%	(90)	——		75%	(224)
20°C	2000	——		8%	(51)	——	
	3000	8%	(63)	61%	(106)	7%	(98)
	4000	33%	(96)	98%	(62)	36%	(213)
	5000	73%	(78)	100%	(40)	77%	(221)
25°C	3000	3%	(80)	36%	(156)	2%	(50)
	4000	8%	(64)	62%	(93)	9%	(100)
	5000	59%	(30)	95%	(100)	61%	(218)
	5500	68%	(90)	100%	(40)	74%	(68)
	6000	93%	(70)	——		89%	(230)

[1] Figures in parentheses indicate total numbers of specimens observed in each case. Fractional percentages omitted, i.e., percentages represent nearest whole numbers.

The lengthy experimental procedure precludes an accurate timing of the nuclear effect, but clearly it reaches a readily measurable magnitude within an hour after the cutting operation. Apparently the nucleus does not directly govern the cycle of sol-gel changes which determines amoeboid locomotion — or otherwise the anucleate specimens could not continue their amoeboid activity. Rather it seems more likely that the nucleus has an effect upon the steady state level of metabolites which contribute energy to the gelation reaction (Landau, Zimmerman and Marsland, '55).

Precisely how the nucleus may influence the sources of metabolic energy is altogether problematical. However, the demonstrated nuclear dependence of the DPN resources of the cell (Cohen, '56) and of phosphate uptake by the cell (Mazia and Hirshfield, '50), and the demonstrated importance of ATP in relation to amoeboid activity (Weber and Portzehl,

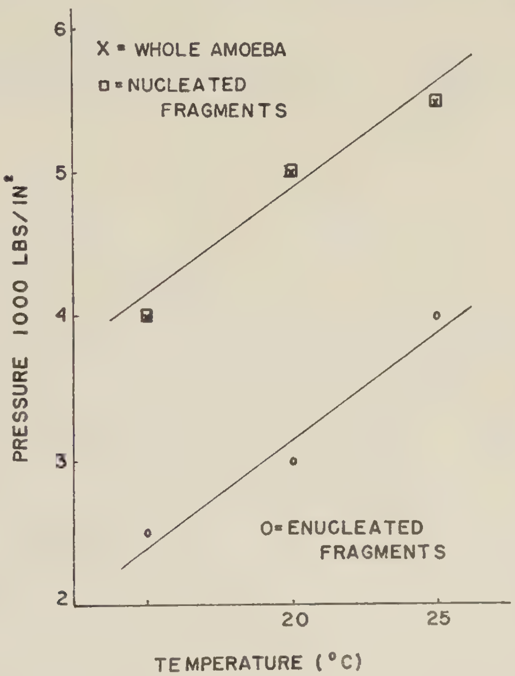

Fig. 1 Comparison between nucleate and anucleate specimens of *Amoeba proteus* in regard to the effect of temperature upon the minimum pressure required to produce total rounding up (solation).

'54) and to other sol-gel determined cell activities (Hoffman-Berling, '54 and Marsland, '56), all tend to suggest that the maintenance of ATP reserves of the cell may be an important factor in the situation.

SUMMARY

Nucleate and anucleate halves of *Amoeba proteus* were compared with each other and with whole (uncut) specimens with

reference to their susceptibility to solation (rounding up) on exposure to high pressures (2000–6000 lb/in.^{2}), at three temperatures (15°, 20° and 25°C). Consistently the nucleated specimens displayed a more firmly set plasmagel structure, since the pressure required to induce rounding up was definitely higher (by about 1500 lb/in.^{2}) at each of the temperatures tested.

The results indicate that the maintenance of the plasmagel structure displays a definite nuclear dependence, probably because it requires metabolic energy.

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ACTION OF CERTAIN ANTICHOLINESTERASES ON THE SPIKE POTENTIAL OF THE DESHEATHED SCIATIC NERVE OF THE BULLFROG [1]

E. R. WHITCOMB, S. L. FRIESS AND J. W. MOORE

National Institute of Neurological Diseases and Blindness and the Naval Medical Research Institute

ELEVEN FIGURES

INTRODUCTION

In a preliminary study (Moore et al., '54) observations were made on the blocking of impulse propagation in the desheathed bullfrog sciatic nerve by a diamine inhibitor of acetylcholinesterase (AChE). The present study is a continuation and expansion of this work using a wider spectrum of AChE inhibitors at various pH levels and concentrations. The relative degrees of blocking of the action potential by the different AChE inhibitors are compared and a correlation attempted in terms of molecular structures and *in vitro* enzymatic inhibitory abilities.

These diamines and their monoquaternary derivatives have previously been rated in terms of their *in vitro* ability to inhibit the purified esterase from electric eel tissue (Friess, '54a, '54b). Their variation in strength of inhibition from member to member was interpreted as a function of molecular structure and the complementary fine structure of the catalytic unit on the enzyme's surface. In the light of this information it was of considerable interest to observe their action on the nerve preparation, since, for example, the studies of Nachmansohn and co-workers (Bullock, '47; Couteaux, '46; Nachmansohn, '47) and Sandow and Kiebel ('52a, '52b) have

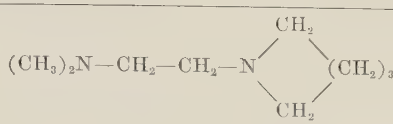
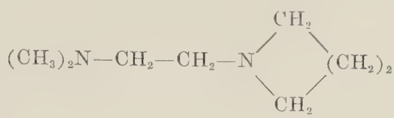
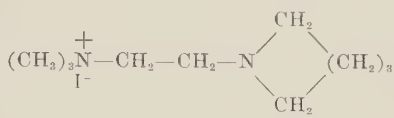
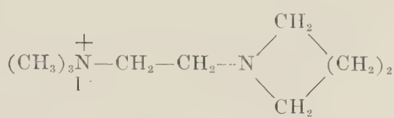
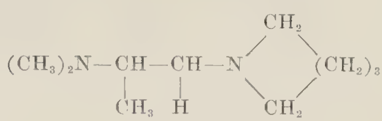
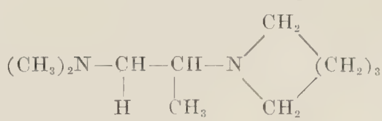
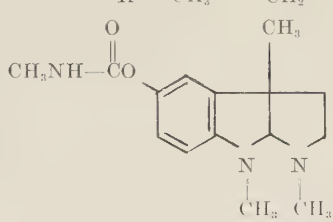
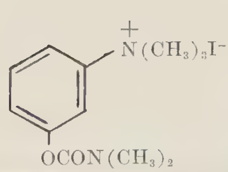
[1] The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department.

called attention to a possible role of the membrane acetylcholinesterase in processes of excitation and conduction in both nerve and muscle. Attenuation of the action potential was used as an index of effectiveness of the inhibitors, as in previous investigations (Bullock, '47; Couteaux, '46; Moore, '54; Nachmansohn, '47; Sandow and Kiebel, '52a, '52b; Wright, '56).

The compounds used in the present study are listed in table 1, together with their dissociation constants for AChE-inhibitor complexes (K_I) as determined previously (Friess and McCarville, '54, '56a). The *in vitro* kinetic observations were made for the most part at pH 7.4, under which condition the diamines I and II would exist essentially as monoprotonated $-\overset{H}{\underset{+}{N}}(CH_3)_2$ species (Friess and Baldridge, '56b) in solution, reverting to the tertiary form $-N(CH_3)_2$ with increase in pH level. The same behavior with change in pH level might be inferred for compounds V and VI in view of the striking constancy of pK_a' values for substituted ethylenediamines noted by Gero ('54). Compounds III, IV and prostigmine (quaternary species, $-\overset{+}{N}(CH_3)_3$) are at least monocharged at all pH levels. However, the ratio of tertiary to monoprotonated species for the compounds eserine, I, II, V and VI will be dependent on the pH level. That this factor must be considered in interpreting the effects of the inhibitors on the nerve is evident from previous observations in the literature. For example, Rothenberg et al. ('48) demonstrated that the tertiary compound trimethylamine was able to penetrate from the external medium into the axoplasm of the giant axon of the squid but that the quaternary acetylcholine ion could not. However, this inability of acetylcholine to penetrate has been questioned recently in the work of Mitchell et al. ('52). Also, Sandow and Kiebel ('52a, 52b) observed in their studies of eserine-veratrine interaction on frog sartorius muscle that in contrast to the dramatic effects of the tertiary drug eserine in altering normal and veratrine re-

TABLE 1

Blocking agents

CODE NO.	SYMBOL	M.W.	STRUCTURE	DISSOCIATION CONSTANTS (K_I) OF AChE-INHIBITOR COMPLEXES
I	DMPi	156.27		$6.4 \pm 0.2 \times 10^{-8}$
II	DMPy	142.24		$4.9 \pm 0.3 \times 10^{-8}$
III	TMPi	298.21		$1.6 \pm 0.1 \times 10^{-8}$
IV	TMPy	284.19		$2.3 \pm 0.1 \times 10^{-8}$
V	α-MeDMPi	171.27		1.00×10^{-5}
VI	β-MeDMPi	171.27		1.04×10^{-5}
	Eserine	272.32		6.1×10^{-8}
	Prostigmine Iodide	350.20		$16. \times 10^{-8}$

sponses, the quaternary compound prostigmine had no effect on these responses.

Accordingly, since charge type appears to have at least some bearing on activity, both tertiary and monocharged compounds were used in the present study.

METHODS

Experimental design

In order to interpret experiments of relatively long duration with some confidence, a good index of the nerve's condition was required. In preliminary experiments action potential electrodes were placed preceding (proximal) and following (distal) the middle (drug) chamber. By taking a ratio (R) of the two action potential values, for a given sweep, any change observed at the distal electrode could be attributed to the solution in the drug chamber. Only those nerves were used which exhibited a constant ratio with passage of time when Ringer solution was in the drug chamber. Figure 1 shows the final experimental arrangement in which differential action potential electrodes were placed preceding and following the middle (drug) chamber so that the impulse

NERVE MOUNTING CHAMBER AND ELECTRICAL SETUP

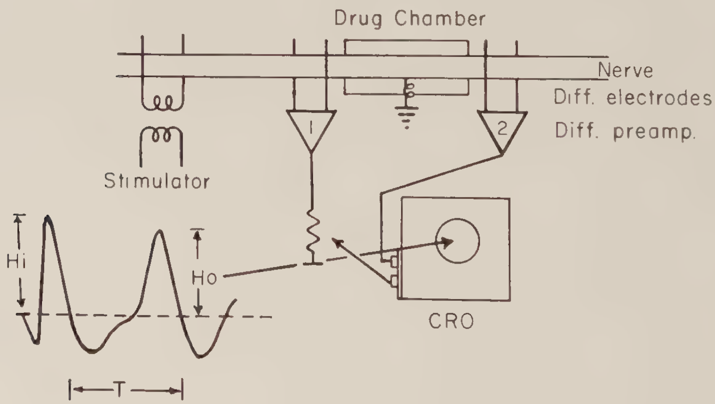


Fig. 1 Diagram of the nerve mounting chamber and its electrical components.

velocity could be determined. The close spacing of these electrodes (2 mm) allowed a good approximation to the temporal derivative to be obtained from the difference in their potentials (Cole and Curtis, '39). The first time derivative goes through zero at the peak of the action potential and this is useful in determining the time at which the peak of the waves passes the mid-point of each differential pair of electrodes. The output of the two differential preamplifiers (Tektronix 122) was fed to a differential input of a cathode-ray oscilloscope (CRO). The gain of each preamplifier was approximately 1,000. The CRO sensitivity was adjusted to give a nearly full scale deflection for the action potential recorded at the distal differential electrodes with Ringer solution in the drug chamber. The proximal action potential deflection was then adjusted with an attenuator between the preamplifier and CRO to give a convenient on-scale deflection.

Figure 2 is a photograph of the plastic chamber and shield box. Hypodermic needles were used as electrodes in the plastic chamber. Banana plugs were fitted to the hypodermic taper joint. Solutions were introduced and removed by means of a hypodermic needle in the bottom of the drug chamber. In changing the solution in the drug chamber, which has a volume of approximately 1 ml, the chamber was flushed twice before the final filling. The moist chambers on each side of the drug chamber were sealed from the middle chamber with stopcock grease and a plastic cover.

The short branches of the sciatic nerve of *Rana catesbiana* were cut close to the main trunk. After girdling at the bifurcation the sheath was removed as one would slip off a rubber glove. The electrical stimulation was increased until response of the "B" fibers was noted on the CRO, thereby insuring that all "A" fibers were being adequately stimulated in order to provide the highest sensitivity in study of conduction decrements produced by drugs. The repetition rate was 1/sec. The experiments were conducted at room temperature (23–28°C).

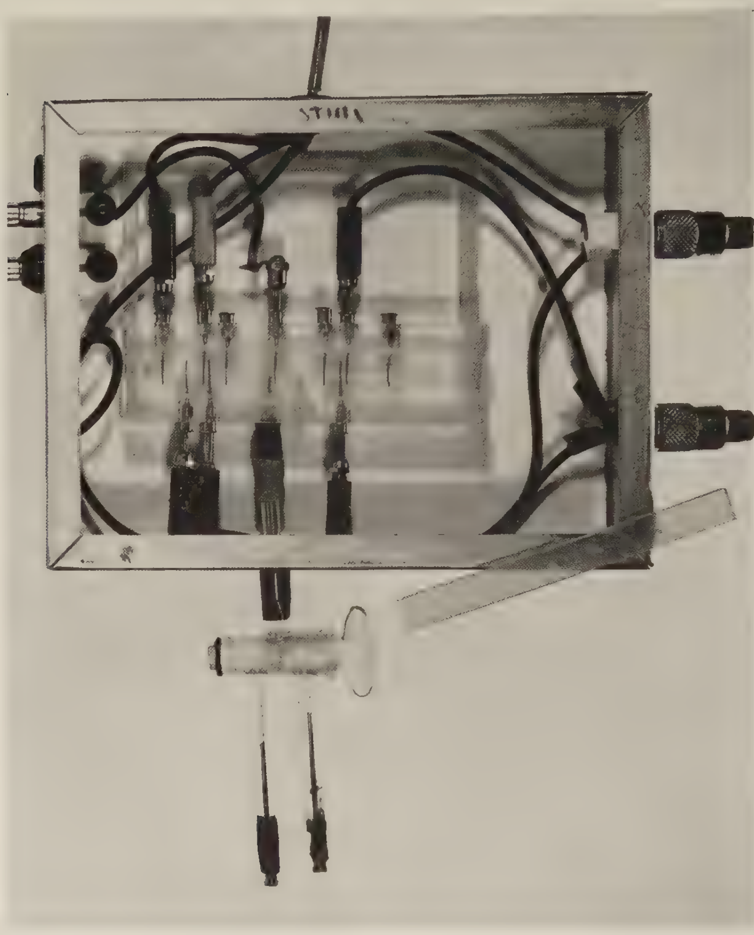


Fig. 2 Photograph of the plastic chamber and shield box.

Solutions

Carbonate and phosphate Ringer solutions were employed. Their constituents and final concentrations are listed in table 2. The stock drug solutions were made at a concentration of 118 mM. Their final dilutions to desired concentration levels were made with Ringer solution. The pH level was then re-adjusted with 1 N HCl or NaOH. Dilution to furnish the 10 mM drug solutions resulted in approximately 10% reduc-

tion of the initial ionic level of the Ringer solution. Eserine sulfate and prostigmine methosulfate stock solutions were made fresh for each experiment. The diamine inhibitors and their stock solutions were stored at 5–10°C.

TABLE 2

Composition of Ringer solutions (initial concentrations, meq/1)

IONS	PHOSPHATE		CARBONATE
	pH 6.7	pH 7.6	pH 8.1
Na^+	116.2	118.8	113.1
K^+	2.6	2.6	2.6
Ca^{++}	.7	.7	.7
Mg^{++}	1.2	1.2	1.2
Cl^-	111.	111.	104.8
$H_2PO_4^-$	3.1	.5	—
HPO_4^{--}	3.1	5.7	—
HCO_3^-	—	—	12.3

Records

In the schematic record shown in figure 1 the heights of the peaks (labeled H_i and H_0) are proportional to the rate of rise of the action potential at the electrodes. The time interval T is the time required for the impulse to pass from the mid-point of the proximal electrodes to the mid-point of the distal electrodes. In a preliminary experiment, figure 3, it was observed that the changes in the ratio H_i/H_0 obtained from differential electrode recordings were proportional to changes in the action potential patterns from monopolar leads at these points. Since this proportionality means that the peak of the action potential and the rate of its rise are linearly related as a function of time, the derivative patterns were used routinely for analysis. A typical set of records is illustrated in figure 4 showing the action potential preceding and following the drug chamber for each sweep. Records were taken periodically while the nerve was in the chamber. In figure 4 the distal action potential can be seen to decrease in amplitude, accompanied by a decrease in velocity of the impulse propagation, as the eserine exerts its effect.

The empirical percentage of blocking (percentage attenuation) of the action potential was obtained from the action potential ratio, R, vs. time curves (see for example figs. 5 and 6). The percentage attenuation for each compound represents the fractional decrease in the action potential produced by the

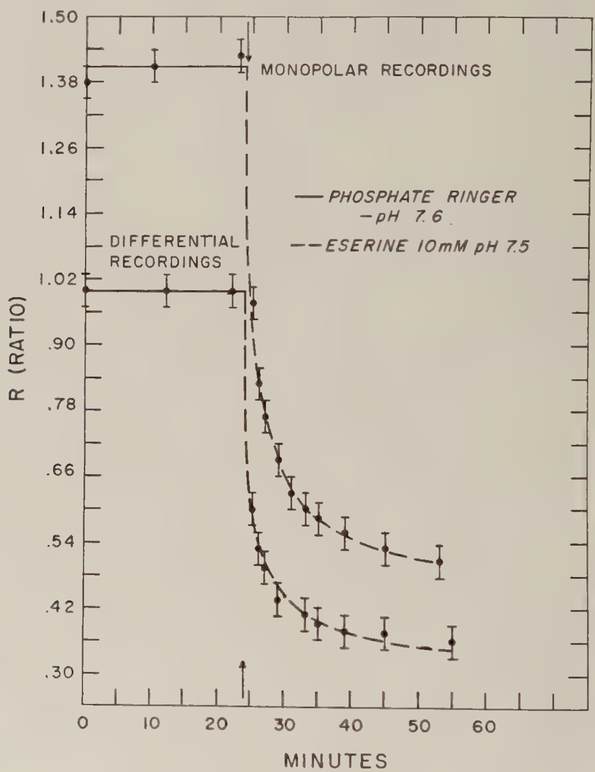


Fig. 3 Comparison of monopolar vs. differential recordings for a nerve under the effect of 10 mM eserine at pH 7.5. Estimated error is indicated.

drug. When $\frac{dR}{dt}$ (obtained by graphical differentiation) was plotted against R, as shown in representative figures 7 and 8, a straight line was obtained for some of the experiments. The slope of this line gave the apparent time constant τ for the overall process of inhibition. The intercept on the ordinate axis gave the extrapolated steady state value for R. The percentage attenuation obtained using the steady state value for

R was quite close to the observed empirical value in nearly every case. The recovery of the action potential after washing is reported as the percentage recovery from the observed attenuation.

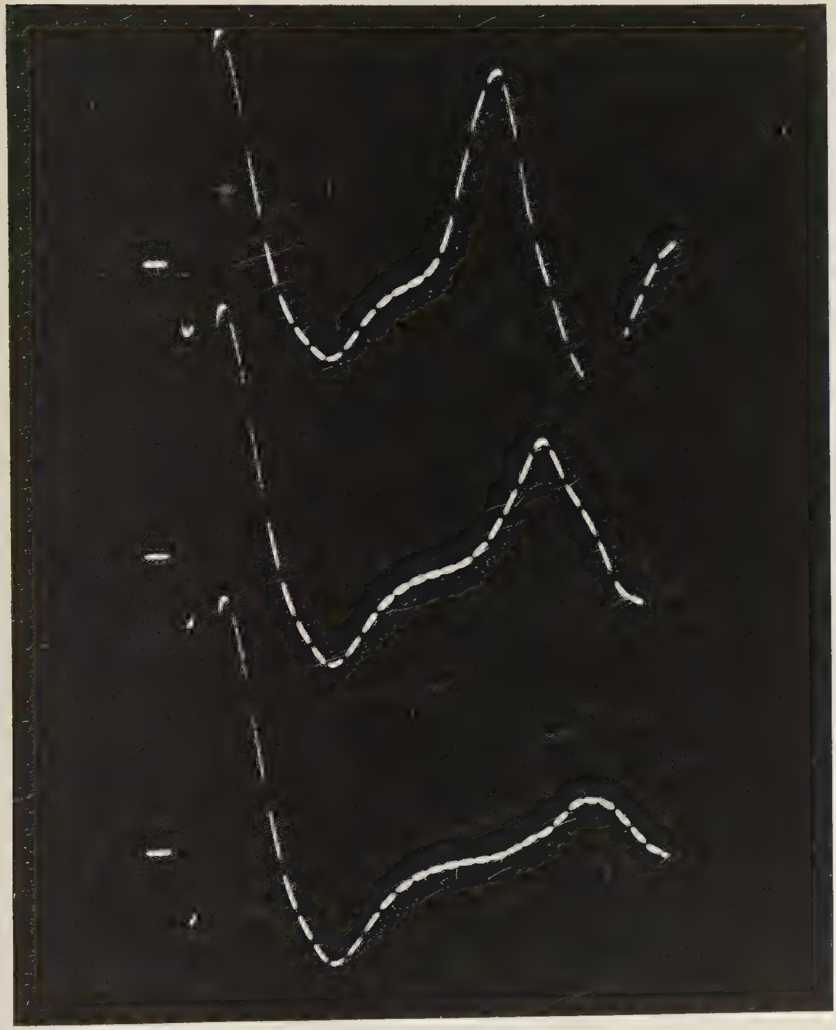


Fig. 4 Action potential preceding and following the drug chamber for each sweep. From top to bottom, tracings made at progressive intervals during treatment of nerve with eserine.

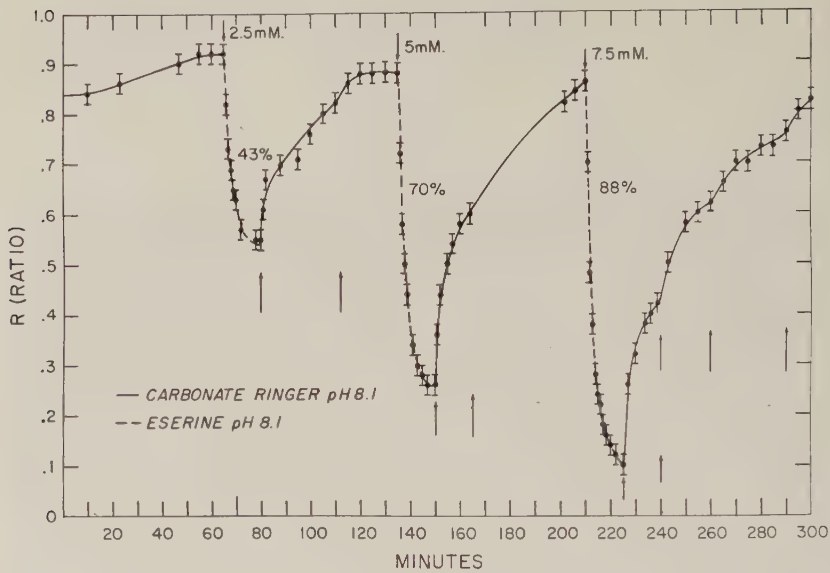


Fig. 5 Action potential ratio R vs. time for the action of eserine on the nerve at pH 8.1. Arrows indicate the points at which solutions were changed to the new ones indicated on the figure. Percentages refer to final attenuations.

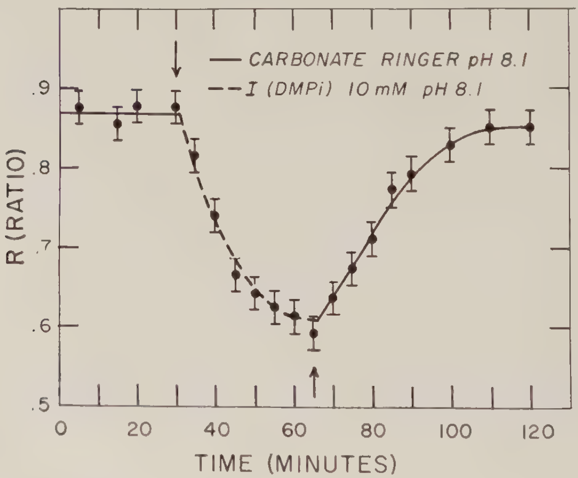


Fig. 6 Action potential ratio R vs. time for the action of 10 mM. DMPi(I) on the nerve at pH 8.1.

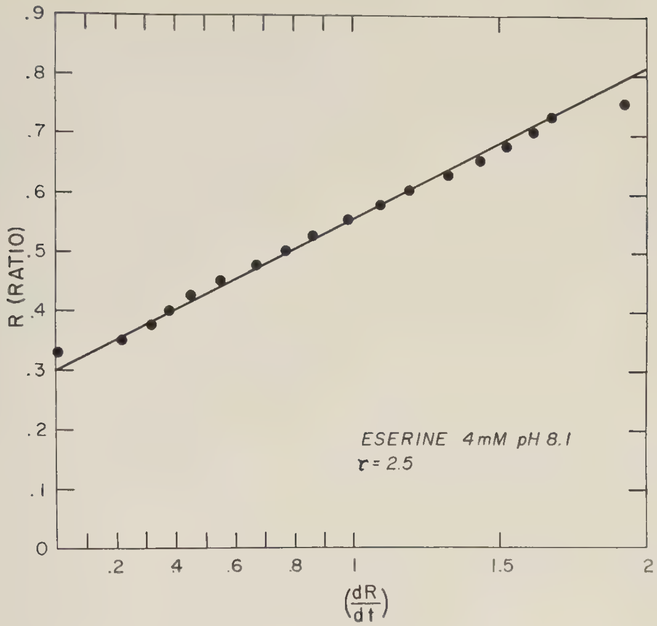


Fig. 7 R vs. $\frac{dR}{dt}$ plot for action of eserine at 4 mM conc. and pH 8.1.

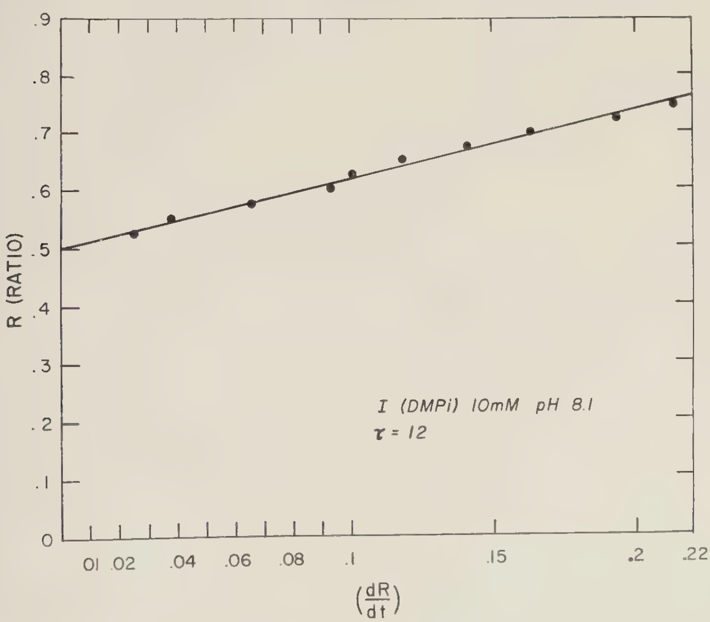


Fig. 8 R vs. $\frac{dR}{dt}$ plot for action of DMPi, 10 mM conc., pH 8.1.

RESULTS

Effect of Ringer solutions

That the Ringer solutions at the three pH levels (6.7, 7.6, and 8.1) did not attenuate the action potential significantly was indicated by the following observations: (1) the action potential of the desheathed nerve preparation showed no attenuation during a 5–6 hour period in Ringer solution at any of these pH levels; (2) no change was observed when a given Ringer solution in the middle chamber was replaced by a different one; and (3) nerves that had been refrigerated for 24 hours at 5–10°C in the various Ringer solutions produced action potential values after desheathing that were within the range given by fresh nerves.

Velocity of propagation

Figure 9 illustrates that the velocity of propagation decreased with increasing attenuation (as given by steady-state

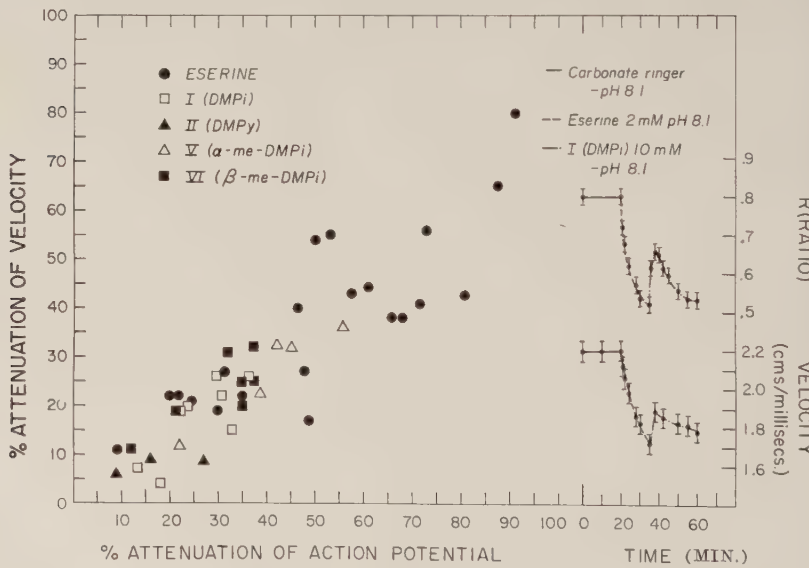


Fig. 9 On left, data showing percentage attenuation of spike velocity vs. percentage attenuation of action potential; on right, sequential action of 2 mM eserine followed by 10 mM. DMPi, with comparative plots of R vs. time and impulse velocity vs. time of contact with drug.

values of R) of the action potential for the AChE inhibitors used.

Effect of KCl

The blocking effect of KCl as a standard agent at 20 mM concentration was determined with this nerve preparation. A period of 15–20 minutes was required to produce complete blockade at this level. Higher concentrations of KCl (25 mM) block the action potential completely in 2–3 minutes.

Eserine

Eserine at various combinations of concentration and pH level was applied to the desheathed nerve. Figure 5 depicts an experiment showing the increase in blocking action with increasing concentration of eserine at pH 8.1. The results of all the eserine experiments are summarized in table 3. The maximum per cent attenuation for a given concentration increases with increasing pH level, and at a given pH level it increases with higher concentration of the drug. These results were obtained with frogs studied at different seasons of the year. When the percentage attenuation was plotted vs. the concentration of tertiary eserine species present in solution a roughly linear relationship was obtained (fig. 10).

TABLE 3

Attenuation by eserine

CONC.	pH	TERTIARY CONC.	MONO-PROTONATED CONC.	MAXIMUM % ATTENUATION	NUMBER OF TRIALS
mM		*mM*	*mM*		
8	6.7	.48	7.52	23	6
8	7.6	1.68	6.32	52	4
6	7.6	1.26	4.74	22	3
8	8.1	3.84	4.16	90	4
7.5	8.1	3.6	3.9	88	1
6	8.1	2.88	3.12	80	3
5	8.1	2.4	2.6	70	1
4	8.1	1.92	2.08	57	4
2.5	8.1	1.2	1.3	40	1
2	8.1	.98	1.02	39	4

From those experiments in which a time constant could be calculated, because the $\frac{dR}{dt}$ vs. R plot was linear, a range of τ values between 2–5 minutes was obtained. However, all such plots were not linear, with the greatest deviations from linearity occurring in runs above and below the 4 mM concentration level at pH 8.1. Recovery of the action potential in Ringer

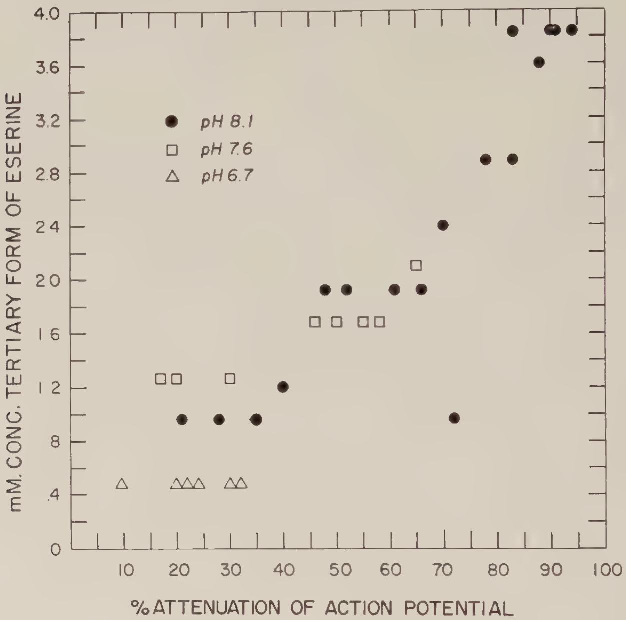


Fig. 10 Data showing correlation of percentage attenuation of action potential with the concentration of free base form of eserine, over the pH range 6.7–8.1.

solution following various percentages of attenuation averaged 87%. Because of the greater and more rapid attenuation produced by eserine as compared with the other inhibitors the contact time employed was less than that used for the diamines, and lower concentrations of drug were applied.

Prostigmine

For prostigmine at 10 mM concentration and pH levels of 6.7, 7.6, and 8.1 no attenuation of the action potential was observed. (See table 4.)

Diamine AChE inhibitors

One concentration (10 mM) of the diamines was used at all pH levels. Table 5 summarizes the data for the action of these diamines, while an experiment with good recovery on washing is shown in figure 6. No blocking action was observed for the quaternary compounds III and IV. With increasing pH level compounds I, II, V and VI produced progressively greater

TABLE 4

Prostigmine attenuation

CONC.	pH	MAXIMUM % ATTENUATION	NUMBER OF TRIALS
mM			
8	6.7	0	1
8	7.6	0	1
8	8.1	0	2

TABLE 5

Attenuation produced by diamines

CONC.	pH	MAXIMUM % ATTENUATION					
		I (DMPi)	II (DMPy)	III (TMPi)	IV (TMPy)	V (α-MeDMPi)	VI (β-MeDMPi)
mM							
10	8.1	25	22	0	0	39	33
10	7.6	16	—	0	0	19	12
10	6.7	8	7	0	0	5	3

attenuation. At pH 8.1 compounds V and VI produced a greater percentage attenuation than I and II, even though the latter display a greater *in vitro* enzymatic inhibitory power. Time constants were obtained in some experiments. At pH 8.1 the time constants for the action of I ranged from 12–15 mins., for V from 7–12 mins., and for VI from 6–11 minutes. The recovery of the action potential in Ringer solution after maximum attenuation at pH 8.1 with drugs was not the same for the various diamines. The average recovery on washing was 63% for I, 5% for II, 26% for V and 55% for VI under these conditions.

Sequential application of eserine and diamines

To determine if a given inhibitor could alter the action of another inhibitor by "conditioning" the nerve, the desheathed nerve was treated with these AChE inhibitors in various sequences. When eserine replaced any of the diamine inhibitors which had produced some attenuation, the eserine further attenuated the action potential to the degree obtained with nerves not previously treated with a diamine. Likewise, when eserine was replaced by a diamine inhibitor, the final degree of attenuation was approximately that which the diamine could produce on a nerve not previously treated with eserine. When eserine had attenuated the action potential to approximately the level obtainable by the diamine, upon substituting the latter the nerve recovered for a few minutes and then returned to the approximate attenuation level characteristic of the diamine (fig. 11a). When eserine had produced a much greater attenuation of the action potential than that obtainable by the diamine, then upon substituting the latter the nerve showed recovery to approximately the attenuation level obtainable by the diamine (fig. 11b).

DISCUSSION

Eserine

It has been demonstrated (table 3) that the effectiveness of eserine increases with increasing pH levels. Actually, there appears to be a roughly linear relationship between the concentration of tertiary form of eserine in solution and the percentage attenuation of the action potential (fig. 10). Eserine has a dissociation constant of 8×10^{-9} at 20°C (Nachmansohn and Wilson, '51) and changes from 99% cation at pH 5.9 to 0.6% cation at pH 10.1. This cation (monoprotonated) has been shown to be more effective than the tertiary form as an AChE inhibitor *in vitro* (Wilson and Bergmann, '50), but with respect to action on nerve, the tertiary form of eserine is apparently more effective. If one now considers the action potential attenuation within the framework of the proposition

(Rothenberg et al., '48; Sandow and Kiebel, '52a, '52b; Wright, '56) that tertiary species can penetrate and act at interior sites whereas quaternary and monoprotonated species cannot, then the eserine inhibition results obtained *in vitro* and those with the nerve preparation are compatible. In this picture the tertiary species can be viewed as penetrating to

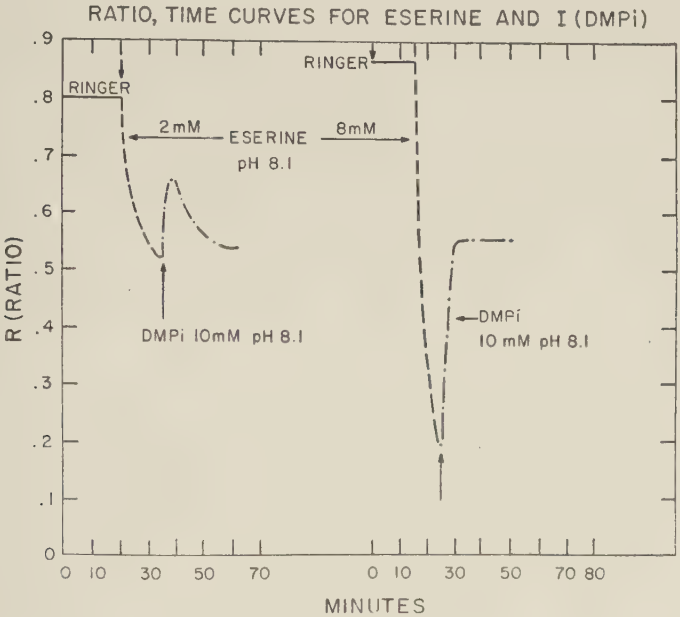


Fig. 11 Sequential experiments with eserine and DMPi at pH 8.1. See text for details.

reactive zones with a driving force dependent on the concentration of tertiary form in solution, resulting in an attenuation of the action potential roughly proportional to this concentration. This does not require that once penetration has occurred, it is necessarily the tertiary species alone that produces the attenuation since the pH level of the site of action will regulate the tertiary-monoprotonated species (base-salt) equilibrium at that point. An additional observation in accord with this general view stems from experiments which indicate that

equivalent concentrations of the tertiary form of eserine produce nearly equivalent percentage attenuation levels on the sheathed and desheathed nerve preparations. Wright ('56) was able to produce an 80–85% attenuation in 80 minutes on a sheathed sciatic nerve using a 20 mM concentration of eserine at pH 7.4. At this pH level approximately 15% of the eserine is in the tertiary form (Wilson and Bergmann, '50). Therefore, at a total eserine concentration of 20 mM the tertiary species concentration would be 3 mM. On the desheathed nerve in the present work, this amount of tertiary form would have produced the same final level of attenuation within 10–20 minutes. Allowing additional time for penetration of the sheath, this comparison also indicates that action on the sheathed nerve by the tertiary form resulted in blockade of a magnitude roughly in proportion to the concentration of the tertiary form of eserine present in solution.

Prostigmine

At a concentration of 10 mM and pH levels 6.7, 7.6, and 8.1 this compound was ineffective in attenuating the action potential. Since this compound is a quaternary ion in solution at all pH levels, its inability to reduce the action potential is in accordance with the possibility (Wright, '56) that only tertiary species can penetrate to the regions actively involved in the attenuation process.

Diamine inhibitors I–VI

From the *in vitro* data (Friess et al., '54, '56a, '56b) these compounds may be grouped into categories with respect to their charge as a function of pH levels and their relative inhibitory abilities vs. isolated AChE.

(1) Charged species

a. Compounds III and IV are permanently charged by virtue of the quaternary nitrogen atom, resulting in at least one unit of positive charge per molecule at all pH levels.

b. Compounds I, II, V [2] and VI [2] are roughly 50% monoprotonated at pH 9.6 and 99% monoprotonated at pH 7.5.

(2) Inhibitory strength

Compounds I, II, III, and IV are roughly equivalent in inhibitory strength *in vitro* with K_I values of the order of 10^{-8}; compounds V and VI are about a thousand-fold weaker, with K_I values in the 1×10^{-5} range.

With respect to action on the sciatic nerve, the quaternary compounds III and IV were ineffective at all three pH levels, while the diamines I, II, V and VI were found to increase in effectiveness with increasing pH. These observations are also in general accord with the possibility (Wright, '56) that quaternary and monoprotonated ions possess little ability to penetrate to reactive sites in the nerve fiber and produce attenuation of the action potential.

Even though the diamines support the thesis that tertiary structure is a requirement for attenuation of the action potential by amine blocking agents, the following series of observations indicates that a more complex mechanism may be involved.

(1) At pH 8.1 compounds I and V furnish roughly the same ratio of monoprotonated to tertiary forms in solution, but I is a more potent *in vitro* inhibitor of AChE (table 1). Assuming that penetration by the tertiary form and inhibition of the tissue-localized AChE results in attenuation of the action potential, then I should have produced a much larger percentage attenuation of the action potential than V. The results in table 5 show that the attenuation of the action potential produced by V is approximately 1.5 times that given by I. At equivalent concentrations, this is a complete reversal of the order expected from *in vitro* strengths.

(2) At pH 7.6 eserine is approximately 79% monoprotonated and a total concentration of 10 mM (2.1 mM tertiary

[2] Actual pK_a' values for these two were not determined but they are taken to be of the same magnitude as for I and II since the values do not change markedly with structure in the substituted ethylenediamine series (Friess and Baldridge, '56b; Gero, '54).

form concentration) would have produced about 60% attenuation (fig. 10). At pH 7.5 compound V should be about 99% monoprotonated on the basis of pK_a' values for I, and on the nerve preparation a 10 mM total concentration of V at pH 7.6 (0.1 mM tertiary form) produced approximately 18% attenuation of the action potential. If both of these compounds had penetrated and produced attenuation by action of the *tertiary* species on tissue AChE only, eserine should have caused a much larger attenuation since it has a greater proportion of tertiary form in solution under the given conditions and its intrinsic enzymatic inhibitory power is greater. On the basis of these observations it is indicated that for equivalent amounts of tertiary form in the bathing medium, eserine would produce only 1/6 the attenuation of compound V. Again this is in wide disparity with expectation on the basis of inhibition of tissue-localized AChE only, following a hypothetical penetration by tertiary forms.

(3) Since the K_I value of the reversibly formed AChE-inhibitor complex in solution is a measure of the ability of the inhibitor to bind the enzyme, it is perhaps a reasonable working assumption that the better the inhibitor (lower K_I) the harder it should be to remove it from its binding with tissue-localized enzyme by washing. With the added supposition that recovery of the nerve depends largely on removal of the inhibitor from the tissue, the recovery from compounds I. II and eserine as strong AChE binders should have been poorer than that evidenced after the actions of V and VI, following a standard sequence of washes of the preparation. In practice, after the inhibitors had exerted their maximum effects at the 10 mM level the recovery obtained after eserine, I and VI was quite good, but poor after compounds II and V, pointing to a more complex or different process than simple AChE-binding and release by washing. These observations on reversal of action with washing do not fit an alternative possibility that ease of rinsing away should parallel ease or strength of action on nerve, assuming that the physical act of washing constitutes a direct reversal of the physical processes of penetration

and action, since compound V for example is quite effective in action and yet recovery on washing is poor.

Action of diamine inhibitors on eserine-attenuated nerves

Figure 11a shows a typical experiment in which the action potential is partially attenuated by eserine before the latter is replaced by a diamine inhibitor capable of producing attenuation. The various parts of the curve can be interpreted in terms of a graphic summation of the recovery process from eserine and the attenuation rate produced by the displacing diamine. In this figure the action potential was first attenuated by eserine to approximately the level attainable by the given concentration of diamine next applied. Following the addition of the diamine solution with the accompanying displacement of the eserine solution, the initial recovery rate from eserine appears to exceed the attenuation rate of the diamine inhibitor, resulting in a partial recovery. But in a few minutes the attenuation rate characteristic of the diamine apparently exceeds the residual recovery rate from eserine and the action potential is reduced once again, this time to approximately the intrinsic level afforded by the diamine. In figure 11b the action potential had been lowered by eserine sufficiently below that attainable by the diamine so that by the time solution replacement had caused the action potential to recover to the attenuation level for the given concentration of diamine, the latter was exerting its maximum effect, resulting in a leveling-off at that attenuation value.

General remarks

The results on the action of the present series of diamines with respect to blockade of conduction in the desheathed sciatic nerve lead to some interesting general conclusions. First, the lack of an overall correlation between *in vitro* AChE-inhibitory strengths of these materials and their graded abilities to depress the action potential in the nerve implies rather strongly that the direct action on nerve is not restricted solely

to inhibition of tissue-localized AChE. Other protein systems could conceivably serve as either highly selective "receptors" (Nachmansohn and Wilson, '51) or as absorbing agents of lower specificity, with net physical and chemical changes on interaction with diamines producing the observed effects on action potential. This view is strengthened by the observation that for those natural and synthetic diamines which do produce blockade, much higher concentrations (10^{2}–10^{4} greater) are required for minimal action than are needed for powerful effect against isolated AChE in solution. Then too, the tissue preparation shows relatively little variation in attenuation response with small changes in molecular structure of diamine (e.g., proceeding from I to II), in contrast to the more sensitive response of AChE in solution.

This last point can be taken as a bit of presumptive evidence for one further speculation on the spatial distribution of AChE in the fiber. If it were assumed that AChE is the only protein involved in the process of blockade via the tertiary diamines, then it is not likely that the major portion of this enzyme could be localized in the outermost surface of the fiber. In this event, one would predict action potential attenuation to be closely correlated with solution AChE-inhibitory properties of the diamines, since surface-bound enzyme might be expected to parallel the behavior of AChE in solution to an appreciable extent. That this is not the case would appear to militate against a postulation of high localization of AChE on the fiber surface, which is essentially in accord with the view of Bullock et al. ('46) on AChE localization in nerve.

The possibility discussed above pertaining to at least partial mediation of impulse blockade by key receptor proteins other than AChE has received considerable treatment in recent years at the hands of Nachmansohn and co-workers ('55). In particular, work on esterase inhibition and blockade of the propagated impulse in the single electroplaque of *Electrophorus electricus* has revealed, as in this study, a remarkable differentiation in the abilities of tertiary vs. quaternary amine derivatives to produce blockade and/or depolarization of the

plaque membrane. The intervention of a "receptor" protein or site into the molecular processes leading to blockade, with or without the concommitant production of electroplaque membrane depolarization, was necessarily included in the general correlation of observations that quaternary derivatives block the spike and depolarize while tertiary structures lead to blockade with no depolarization. These results on the electroplaque differ in one significant respect from the frog sciatic observations presented here, however, in the sense that quaternary derivatives have no effect on blockade in the sciatic preparation as contrasted with their potent action (Nachmansohn, '55) against the electroplaque.

SUMMARY

1. The action of eserine, prostigmine, and of a series (I–VI) of substituted ethylenediamine derivatives on attenuation of the action potential in the desheathed frog sciatic nerve has been studied.

2. The potency of these materials in attenuating the action potential bears no simple relationship to their relative inhibitory actions against isolated, purified acetylcholinesterase. However, it does appear that quaternary derivatives of these amines, and perhaps positively charged species in general, may be relatively incapable of penetrating the fibers to the sites of production of attenuation, in contrast to tertiary forms which are effective in producing blockade. Attenuation of action potential also appears to be accompanied by a decreasing velocity of impulse propagation.

3. For eserine studied over the pH range 6.7–8.1, the degree of attenuation produced is a roughly linear function of the percentage of eserine tertiary form in the bathing solution.

4. Prostigmine fails to block conduction at all pH levels studied.

5. Data with respect to compound V indicate that at comparable levels of the tertiary base species in solution, V pro-

duces a greater attenuation that eserine, even though it is intrinsically weaker in its *in vitro* inhibition of acetylcholinesterase.

6. For those inhibitors which produce attenuation of the action potential, recovery of the nerves on washing with buffer is not a simple function of the binding power (*in vitro*) for AChE. Both weak and strong enzyme inhibitors can lead either to good recovery on washing (eserine, I and VI) or poor recovery (II and V). Preliminary conditioning of the nerve with eserine, followed by washing, does not appear to affect the subsequent responses to the other diamines.

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A COMPARISON OF THE ACTIVITY OF NORMAL AND ATYPICAL PIGMENT CELLS FROM SWORDTAILS, PLATYFISH AND THEIR HYBRIDS AGAINST CERTAIN MELANIN PRECURSORS [1]

DOUGLAS G. HUMM AND JANE H. HUMM
Department of Zoology, University of North Carolina, Chapel Hill, N. C.

ONE FIGURE

The first reported observation on pigmented tumors resulting from the hybridization of two species of *Xiphophorus* was made by Häussler ('28). Since that time, Gordon ('27, '31, '51) has extensively analyzed the inheritance of the pigmented tumors and has been able to show that the occurrence of the tumor is correlated with the presence of macromelanophores in the platyfish. He was further able to demonstrate that the representation and distribution of the macromelanophores was controlled in the platyfish by 5 sex-linked genes, Gordon ('48).

The melanophores of the swordtail, platyfish and their hybrids make a very useful tool for the study of the etiology of melanotic fish tumors. The normal macromelanophores of the platyfish, inherited as a simple Mendelian dominant, are never malignant and can be obtained in sufficient quantity to make accurate analysis of their metabolism possible by the use of micro-methods. After hybridization has occurred, the heterozygous macromelanophores grow and migrate much more rapidly than the corresponding cells of the platyfish. As far as can be told by the appearance of the cells, the hybrid macromelanophores are identical with the normal macromelan-

[1] The research reported in this paper was supported by U.S.P.H.S. Grant No. 1717 from the National Cancer Institute.

ophores of the platyfish. However, as indicated by their changed growth rate, these cells are physiologically affected by hybridization. Comparisons between these cell types might give an indication as to what metabolic changes have accompanied the profound change in the growth and migratory habits of these cells.

The hybrids produced by the cross between *Xiphophorus helleri* (the swordtail) and *X. maculatus* (the platyfish) are fertile. These hybrid animals may be backcrossed to the swordtail. Pigment cells obtained from these backcrosses have greater chromosomal heterogeneity than do the simple hybrids. Those animals which carry a gene for macromelanophore production will almost without exception develop a melanoma as young fish. However, since the multiplication and spreading of normal appearing macromelanophores always precedes the formation of a tumor, it is possible to obtain, prior to the formation of a malignancy, samples of backcrossed macromelanophores in sufficient quantity to permit an analysis of their metabolism. The genetic relationship between normal macromelanophores, hybrid mecromelanophores and backcrossed macromelanophores is shown in the following diagram.

The pigmentation of the hybrid was always less intense than that of the "backcross." However, tumors occured with fair frequency in older hybrid fish. Thus, two different tumor tissues were available for analysis: the hybrid tumor, occurring usually in an older fish, and the "backcrossed" tumor, which occurred early in the life history of the host with the correct genetic background. The genetic relationship between these two populations of cells can be inferred by reference to figure 1.

The question arose as to whether the effect of the hybridization was manifested by a change in the physiological environment of the host, which presented a more favorable growth medium for an essentially normal cell, or whether the interaction of the two sets of genes resulted in the production of pigment cells which were morphologically and physiologi-

cally changed in such a manner that additional hybridization eventually produced a malignancy.

In other experiments, reported elsewhere, Marcus and Gordon ('54) and Humm, Clark and Humm ('58) attempted to

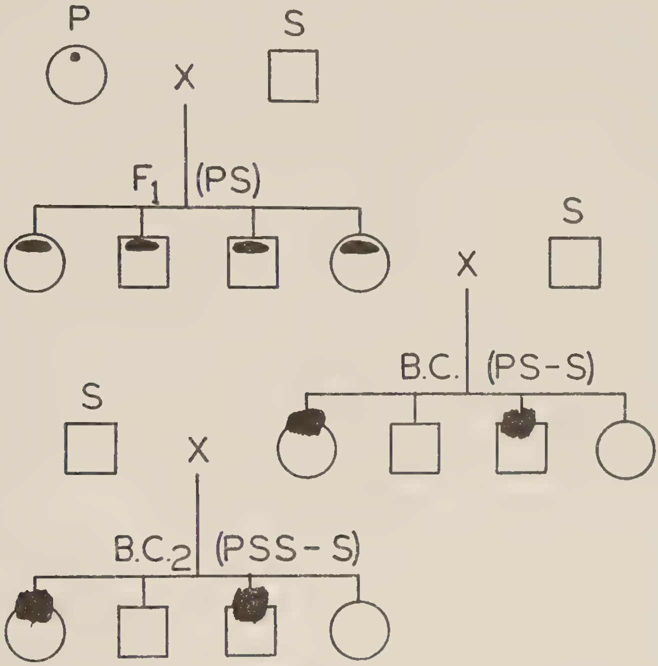

Figure 1

Explanation of symbols. P, Platyfish; S, Swordtail; F_1(PS), Platyfish swordtail hybrid; BC (PS–S), Offspring of a F_1(PS) hybrid and a swordtail; BC_2 (PSS-S), Offspring of a B.C. hybrid and a swordtail; Circle, female; Square, male; Black dot, spotted dorsal macromelanophore gene (Sd) patter in platyfish; Black bar, intensified spotted dorsal gene pattern; Frilled black bar, pigmented outgrowth, product of interaction with growth modifying factors of the swordtail; result, melanosis or melanoma in hybrid.

analyze by transplantation the relationship between the genotype of the host and the survival of grafted tumors. Despite immunological responses, which indicated the probable presence of several tissue specific antigens, it was found that the hybrid presented a more favorable environment for the growth of tumor cells than did either the swordtail or the platyfish.

Survival of grafts was better in macromelanophore bearing platyfish than in swordtails.

As a first step in the investigation of the metabolism of pigmented fish tumors and their precursor cells, Humm and Clark ('55) analyzed the activity of the hyperpigmented premelanoma cells as well as various regions of the fish melanoma against tyrosine, DOPA, and several other substrates. The hyperpigmented cell showed a low endogenous (no substrate) respiration and was moderately active against tyrosine and DOPA. The portion of the tumor which had invaded the subcutaneous muscle layers also showed a low endogenous respiration and was moderately active against tyrosine but showed a considerable activity against DOPA. These cells were regarded by Reed and Gordon ('31) as the causative agents of the tumor growth. The secondary growth of the tumor as an overgrowth at the tumor site, was also analyzed. These cells showed a very much greater endogenous respiration but considerably less activity against DOPA, although relatively more activity against tyrosine.

On the basis of the observed development of the tumor, Reed and Gordon ('31) suggested that, following the hyperplasia of the pigment cells, the invasion of the subcutaneous tissues of the fish represented a secondary stage in the development of the tumor, followed by the appearance of the overgrowth. Humm and Clark ('55) suggested that the great differences in metabolism exhibited by the hyperpigmented cells as compared with the overgrowth tissue represented a manifestation of basic physiological changes which might be correlated with the malignancy of the cells.

On the basis of the above observations it was necessary to investigate the metabolism of normal macromelanophores in order that a comparison might be made between the metabolism of these normal cells and the abnormal cells already analyzed.

Following the clue that as the process of hybridization continues in these fish, pigmentation becomes more intense, the

substrates selected were those primarily concerned with the production of melanin pigments.

MATERIALS AND METHODS

The original stock of the fish used in these experiments were obtained from the Genetics Laboratory of the New York Zoological Society at the American Museum of Natural History contributed through the courtesy of Dr. Myron Gordon, to whom the authors express their sincere appreciation. These fish have been carried through many generations in this laboratory.

The platyfish used as donors for normal macromelanophores were of two genotypes. Fish bearing the sex-linked gene "nigra" were most frequently used, since this gene when homozygous results in an abundant macromelanophore pigmentation on the sides of the platyfish and one fish produces enough macromelanophores for an analysis. Since most of the tumor-bearing fish in the colony arose from platyfish carrying the "spot-dorsal" gene, normal cells were also obtained from the pigmented spot in the dorsal fin which is characteristic of this genotype. No differences were observed between the pigment cells obtained from these two locations.

Hybrid macromelanophores were obtained from hybrids between dorsal spotted platyfish and the wild type swordtail. Reciprocal crosses were used indiscriminately, since it did not seem to affect the cells if the macromelanophore gene was donated by the male or the female. In obtaining backcrosses the cross was always made to the swordtail since experience has shown that this produces more abundant tumor growth. In either case regions of skin were selected which showed hyperpigmentation, but which were completely free of the very characteristic powdery chocolate-brown appearance of the melanoma overgrowth.

Melanoma overgrowths, for comparison with the normal and hyperpigmented tissues, were selected from advanced tumor growths, either from first generation hybrids [P(Sd/Sd) $\times$ S(+/+)] or from backcrossed hybrid fish.

To estimate the effect of hybridization upon the macromelanophore controlling genes as compared with the effect of hybridization on the organism as a whole, it was necessary to select a control tissue which might normally be expected to be active against the melanin precursors but which did not become mitotically hyperactive or malignant upon hybridization. Since the micromelanophore population of these fish answered both these requirements, it was selected as a tissue upon which the effect of hybridization could be measured without the possible complication of special effects due to the macromelanophore controlling genes. These cells occur in considerable numbers on the surface of both platyfish and swordtails, but are particularly numerous in the "sword" of the male swordtail. The black pigmentation of this secondary sexual adornment is exclusively due to micromelanophores. Furthermore, the male hybrid fish frequently possesses a short sword, similarly pigmented. Accordingly, these cells were obtained from normal wild type swordtails and hybrids in sufficient quantity and tested against tyrosine, DOPA, phenylalanine and tryptophane.

The analyses of the activities of the tissues against the 4 substrates were carried out in a Cartesian Diver apparatus (Boell and Needham, '39; Holter and Linderstrøm-Lang, '43) with the substrate being added to the respiring tissue after the method of Anfinsen and Claff ('47).[2] The tissues were suspended in one of two media. Krebs-Ringer phosphate at pH 7.2 was used for the majority of the experiments, although no difference in oxygen consumption was observed when Niu's amphibian embryo solution was employed instead.[3]

[2] A detailed description of the diver loading technique has already been published (Humm and Clark, '55).

[3] Niu's Solution; grams required per liter of solution; pH 7.4–7.5.

Solution A		Solution B	
NaCl	3.4	Na_2HPO_4	0.11
KCl	0.05	KH_2PO_4	0.02
$Ca(NO_3)_2 \cdot 4H_2O$	0.08		
$MgSO_4$	0.10		

Solutions A and B may be sterilized separately by autoclaving but must be cold before mixing.

At the end of the experiments, the tissues were removed from the diver and their nitrogen content analyzed by the method of Boell and Shen ('54) and all results given in the tables are in terms of $Q_{O_2}(N)$.

RESULTS

The cell types used in these experiments fell logically into 4 groups:

I Normal or normal-appearing macromelanophores.
II Normal or normal-appearing micromelanophores.
III Melanoma overgrowth cells.
IV Non-pigmented skin used as controls.

The aerobic oxidations of tyrosine, dihydroxy phenylalanine (DOPA), phenylalanine and tryptophane were tested in the presence of these various types of cells.

I. Normal or normal-appearing macromelanophores

These cells were divided into three groups depending upon their source.

(a) Normal platyfish macromelanophores.

(b) Normal-appearing hybrid macromelanophores, although their number and distribution indicated a hyperactivity both in migration and multiplication.

(c) Normal-appearing macromelanophores from the progeny of a hybrid backcrossed to the swordtail. In these experiments cells were chosen which showed no tendency to invade and lacked the characteristic chocolate-brown pigmentation of the melanoma overgrowth.

As can be seen from a consideration of table 1, there was a general tendency for increasing hybridization to result in an increased activity in the pigmented cells. This can be seen very clearly if the "endogenous" respirations of these various cell types are compared. There was considerable overlap in the ranges of endogenous oxygen consumption, possibly due in part to errors in the nitrogen determinations which were used in arriving at the $Q_{O_2}(N)$. In addition the tissue samples could not, of course, be completely homogeneous as to cell type.

Hybridization also had a considerable effect on the ability of the cells to metabolize tyrosine. Apparently, whatever systems were involved in the oxidation of this substrate in these cells were enhanced by the introduction of swordtail chromosomes.

The addition of phenylalanine to the respiring pigment cells resulted in an increased oxidation. This increase was not affected by the degree of hybridization.

TABLE 1

Macromelanophores

Oxidation of added tyrosine, DOPA, phenylalanine and tryptophane in the presence of various types of macromelanophores. Suspending medium, Krebs-Ringer phosphate, pH 7.2; gas mixture-air; temperature 30°C; final concentration of DOPA 16×10^{-5} M, all other substrates 8×10^{-5} M. All data reported as Q_{O_2} (N).[1]

SUBSTRATE		PLATYFISH MACROMELANOPHORE	PLATYFISH × SWORDTAIL MACROMELANOPHORE	(PLATYFISH × SWORDTAIL) × SWORDTAIL MACROMELANOPHORE
None	Average	10.4	13.3	16.8
	Range	2.2–17.6	7.2–19.2	6.2–29.1
	Detn.	28	21	20
Tyrosine	Average	10.2 (10%)	14.1 (18%)	21.4 (42%)
	Range	4.7–17.6	11.0–18.3	10.5–29.7
	Detn.	8	8	6
	Ratio[2]	2/5	3/3	2/2
DOPA	Average	9.1 (15%)	19.6 (29%)	22.2 (1%)
	Range	2.2–12.6	14.9–23.2	17.9–26.3
	Detn.	10	8	5
	Ratio[2]	2/5	2/3	0/2
Phenyl-alanine	Average	8.4 (17%)	16.0 (17%)	16.8 (19%)
	Range	4.5–12.9	10.1–19.1	10.9–28.5
	Detn.	10	6	7
	Ratio[2]	3/3	1/2	2/2
Trypto-phane	Average	12.8 (21%)	23.1 (64%)	13.3 (1%)
	Range	7.5–15.7	9.8–35.7	11.1–15.0
	Detn.	11	6	8
	Ratio[2]	3/4	2/2	0/3

[1] The percentages given in parentheses represent an average of per cent stimulation determined from each vessel with respect to its own endogenous respiration.

[2] These ratios represent the number of animals whose tissues respond to substrate addition/total number of animals.

The oxidation of DOPA presents an anomalous situation in that an increase in activity against this substance occurs between the normal platyfish macromelanophore and the first generation hybrid cells. However, in the "normal" macromelanophores from the backcross this value decreased to zero. The oxidation of DOPA in the presence of cells was difficult to analyze. The substance in solution was very sensitive to small changes in pH and minute concentrations of heavy metals, principally copper. It is interesting to note that in the 6 DOPA determinations recorded in the case of the backcross macromelanophores, not only did the tissues not respond to the addition of DOPA (although the control vessels did) but on the contrary there was a considerable decrease in the oxygen consumption of the tissue after the addition of the substrate. This inhibition was not observed in the other types of tissues examined and reported here.

A second inconsistency in table 1 appears in the tryptophane file of the backcross column. The effect of one hybridization upon tryptophane oxidation was a 45% increase in activity. The effect of a second introduction of swordtail genes was to reduce the activity practically to zero.

II. Normal or normal-appearing micromelanophores

Two groups of these cells were tested.

(a) Normal swordtail micromelanophores

(b) Hybrid (swordtail × platyfish) micromelanophores

The activity of these cells was measured against the various substrates tested and reported in this paper since it was known that these cells are in certain cases transmitted to the hybrid but in no case do they become melanomatous. It was further felt that since these cells were also pigment cells they might contain enzymes similar if not identical to those found in the macromelanophore. It was thought that by a comparison between the macro- and micro-melanophore it might be possible to establish whether or not the action of hybridization was occurring by direct action on the pigmentary enzymes.

TABLE 2

Micromelanophores

Oxidation of added tyrosine, DOPA, phenylalanine and tryptophane in the presence of various types of micromelanophores. Suspending medium, Krebs-Ringer phosphate, pH 7.2; gas mixture-air; temperature 30°C; final concentration of DOPA 16×10^{-5} M, all other substrates 8×10^{-5} M. All data reported as Q_{O_2} (N).[1]

		Substrate				
		None	Tyrosine	DOPA	Phenylalanine	Tryptophane
Micro-melanophore Swordtail	Average	15.7	14.8 (22%)	14.4 (2%)	18.9 (14%)	15.9 (0%)
	Range	15.7–30.1	9.2–20.6	9.7–25.4	9.4–36.6	7.7–27.5
	Detn.	22	6	7	7	5
	Ratio[2]		2/3	1/3	2/3	0/2
Micro-melanophore Swordtail × platyfish	Average	15.1	15.1 (5%)	12.2 (0%)	13.8 (0%)	14.5 (7%)
	Range	10.9–28.9	10.3–22.4	7.4–15.1	11.9–16.4	11.9–16.5
	Detn.	12	4	4	4	4
	Ratio		1/2	0/2	0/1	1/1

[1] The percentages given in parentheses represent an average of per cent stimulation determined from each vessel with respect to its own endogenous respiration.

[2] These ratios represent the number of animals whose tissues respond to substrate addition/total number of animals.

It can be seen from a consideration of table 2 that hybridization did not cause a great deal of respiratory stimulation in these cells. As far as endogenous respiration was concerned there is a small increase (9%) which may be significant, but larger numbers of these determinations should be done. Both tyrosine and DOPA oxidation appear to have declined as the animals were hybridized. But again the numbers of the animals were small.

In any event despite the small numbers of animals it can be seen that there was no marked increase in the respiratory activity of these pigment cells after hybridization.

It may be presumed from the above table that any effect upon the pigment cells was restricted to action upon the macromelanophores.

III. Melanoma overgrowth cells

Table 3 was constructed to compare the metabolic activities of tumors which occurred in the first generation hybrid with tumors occurring in the progeny resulting from a backcross of the hybrid to the wild type swordtail. First-generation tumors were not very common in these stocks, but when developed they appeared identical to the tumors occurring in the backcrossed fish.

It can be seen that the "no substrate" oxygen consumption of these two tumor tissues were very similar. Reference to table 1 will show that the endogenous respiration of these cells was about intermediate between that of the hybrid macromelanophore and the backcross macromelanophore. The general pattern of oxidation against the 4 substrates tested in these experiments showed the backcross tumor cells to have a greater activity toward tyrosine and phenylalanine while their activity toward DOPA and tryptophane was about the same as that of the hybrid melanoma.

IV. Non-pigmented skin

Since all of the tissue samples used in these experiments, with the possible exception of the melanoma overgrowth, con-

TABLE 3

Melanoma overgrowth cells

Oxidation of added tyrosine, DOPA, phenylalanine and tryptophane in the presence of melanoma overgrowth cells (melanocytes). Suspending medium — Krebs-Ringer phosphate, pH 7.2; gas mixture-air; temperature 30°C. Final concentration of DOPA 16 $\times$ 10^{-5} M, all other substrates 8 $\times$ 10^{-5} M. All data reported as Q_{O_2} (N).[1]

		SUBSTRATE				
		None	Tyrosine	DOPA	Phenylalanine	Tryptophane
Swordtail $\times$ platyfish melanomatous tissue	Average	15.7	17.7 (5%)	12.8 (18%)	14.0 (6%)	16.8 (10%)
	Range	7.1–26.3	11.0–25.1	10.4–17.8	5.5–18.3	7.2–22.0
	Detn.	18	6	5	6	4
	Ratio[2]		1/2	2/2	1/2	1/1
Swordtail $\times$ (swordtail $\times$ platyfish) melanomatous tissue	Average	14.7[3]	20.9 (48%)[3]	16.0 (30%)[3]	15.9 (33%)	16.3 (19%)
	Range	3.3–31.7	10.2–33.1	13.0–17.0	5.8–26.0	4.8–31.0
	Detn.	27	9	4	12	10
	Ratio		4/4	2/2	2/4	2/2

[1] The percentages given in parentheses represent an average of per cent stimulation determined from each vessel with respect to its own endogenous respiration.

[2] These ratios represent the number of animals whose tissues respond to substrate addition/total number of animals.

[3] Data from Humm and Clark ('55).

tained skin, it was necessary to exclude this tissue as a possible source of the activities being measured in the diver. Areas of skin were selected from all the types of crosses reported here and tested against the 4 substrates. Table 4 presents a summary of these experiments.

DISCUSSION

It is probably somewhat naive to search for a unit change in enzyme activity as a causal agent in the production of an atypical growth. In the melanomatous fish, however, the production of melanoma is associated always and only with macromelanophores, and although it is quite probable that none of the changes in activity have any causal bearing upon the formation of melanotic tumors in fish, the correlation between the increase in endogenous respiration and the increase in the activity of these cells against tyrosine should not pass without comment.

From the data at present available it is not possible to decide directly whether the action of these cells against tyrosine is due to tyrosinase, tyrosine decarboxylase coupled with tyramine oxidase or aerobic or anaerobic (cytochrome-coupled) *l*-amino acid dehydrogenase. The latter two enzymes have such a broad spectrum of activity it is hard to believe that were they present in these cells, they would not act on tryptophane and DOPA. In as much as no activity occurs against these two substrates in the "normal" backcrossed macromelanophore it seems likely that the system being measured in the presence of tyrosine does not include either of these enzymes.

The evidence seems to favor the presence of tyrosine decarboxylase, since in most tissues where it has been determined, tyrosinase activity is very closely coupled with DOPA oxidase activity. In these cells there seems to be a separation of these two activities.

TABLE 4

Unpigmented skin

Oxidation of added tyrosine, DOPA, phenylalanine and tryptophane in the presence of unpigmented skin. Suspending medium, Krebs-Ringer phosphate, pH 7.2; gas mixture-air; temperature 30°C; final concentration of DOPA 16×10^{-5} M, all other substrates 8×10^{-5} M. All data reported as Q_{O_2} (N).[1]

		SUBSTRATE				
		None	Tyrosine	DOPA	Phenylalanine	Tryptophane
Swordtail × swordtail unpigmented skin	Average	13.3	11.3 (0%)	13.2 (0%)	11.8 (10%)	14.6 (3%)
	Range	7.3–24.5	11.3–11.4	10.1–18.5	9.4–13.9	7.5–26.0
	Detn.	10	2	3	2	3
	Ratio[2]		0/1	0/1	1/1	1/1
(Swordtail × platyfish) × swordtail unpigmented skin	Average	15.3	15.9 (3%)	13.0 (10%)	18.5 (48%)	15.6 (0%)
	Range	2.9–26.3	7.1–23.2	11.5–14.1	5.8–35.0	11.5–22.7
	Detn.	17	5	3	6	3
	Ratio		1/3	1/1	3/3	0/1
Platyfish × platyfish unpigmented skin	Average	24.3	23.9 (3%)		28.9 (41%)	
	Range	15.6–35.7	17.2–34.7		18.4–48.0	
	Detn.	10	5		5	
	Ratio		2/3		2/3	

[1] The percentages given in parentheses represent an average of per cent stimulation determined from each vessel with respect to its own endogenous respiration.

[2] These ratios represent the number of animals whose tissues respond to substrate addition/total number of animals.

The widespread occurrence of tyramine oxidase would make the following two step oxidation quite possible. The second step requires the utilization of ½ mole of oxygen.

$$\text{Tyrosine} \xrightarrow[\text{decarboxylase}]{\text{tyrosine}} \text{Tyramine,}$$

$$\text{Tyramine} \xrightarrow[\text{oxidase}]{\text{Tyramine}} 2(\text{p-hydroxy phenyl) acetaldehyde.}$$

The disappearance of DOPA and tryptophane activity from the backcrossed normal-appearing macromelanophores and its reappearance in the tumor cells is a challenging observation. It opens a possibility which should be explored, namely that in the backcrossed hybrid fish there may exist two distinct populations of pigment cells derived from the macromelanophores of the platy parent.

In the selection of fish for use in the preparation of backcrossed normal macromelanophores, care was taken that the cells were removed from areas which were not contaminated by melanoma cells. It was natural to favor as donors those fish which had no tumors at all, thus ensuring freedom from melanoma cells and saving the melanoma bearing fish for other determinations.

If the assumption was made that tryptophane oxidase activity segregated independently from tyrosine oxidation, consideration of the crosses involved in the production of the backcrossed fish would show that, assuming the platyfish to be homozygous for both tyrosine activity and tryptophane action, the hybrid would possess both, and apparently both were increased by the admixture of swordtail genes. However, if the swordtail were assumed to bear intensifier factors but no gene for tryptophane oxidation then the backcrossed fish population arising from a hybrid heterozygous for both tyrosine and tryptophane oxidation when backcrossed to the swordtail would have 4 different types of offspring:

¼ pigmented fish active against both tyrosine and tryptophane.

¼ pigmented fish active against tyrosine but not active against tryptophane.

¼ non-pigmented fish not active against tyrosine, no activity against tryptophane.

¼ non-pigmented fish not active against tyrosine but theoretically should be active against tryptophane.

It should be pointed out that by no means all the pigmented backcrossed fish in a given brood produce tumors. It is intriguing to speculate that perhaps the selection method outlined in the preceding paragraphs has actually resulted in singling out those fish which do not possess tumors and which also lack activity against tryptophane and probably DOPA. Thus in the backcross two populations of macromelanophore-related pigment cells may exist. The first group of these cells might lack genes for activity against tryptophane and DOPA, be active against tyrosine and phenylalanine and have a high endogenous respiration. They would also be normal appearing macromelanophores. The second group would be active against tryptophane, DOPA, tyrosine and phenylalanine, would have a lower endogenous respiration and be premelanoma cells and would lead to the production of a tumor.

At this point it should be mentioned that the very considerable similarity between that activity against DOPA and tryptophane suggests strongly that these two oxidations are inherited as a unit. The possibility should not be overlooked that they are on the same chromosome. It can further be argued that the tyrosine respiration is not linked to either tryptophane or DOPA and is probably on a separate chromosome.

It can be seen that the augmented endogenous respiration after hybridization does not manifest itself in the micromelanophore population of either the swordtail or the hybrid. Likewise the micromelanophores of these two related animals show no increased activity against tyrosine after hybridization. This is an interesting observation inasmuch as it implies (as Gordon, '27, has already shown) that these two groups of cells are inherited independently and that the two tyrosine-oxidizing enzyme systems are not related. More than that, however, the observation implies that in the case of the

macromelanophore gene the effect of hybridization must be local, perhaps restricted to a chromosome and possibly dignified by the term allele, rather than a generalized effect on the cell, e.g., stimulating the activity of all tyrosine oxidation regardless of where it is found.

It was deemed of interest to determine whether or not the endogenous metabolism of the hybrid fish was in general elevated over that of the swordtail or platyfish tissues. Liver, brain and heart muscle tissue were tested from swordtails, platyfish and their hybrids. Preliminary results indicated that there was no considerable difference in endogenous oxygen consumption among these tissues. Accordingly it appeared very likely that the elevated endogenous oxygen consumption observed from hybrid macromelanophores was a specific change limited to those cells. It remains for further experimentation to determine whether or not this progressive change represents a first step toward malignancy.

SUMMARY

Nine classes of pigment cells from platyfish, swordtails and the platyfish-swordtail hybrids were tested for their activity against tyrosine, DOPA, phenylalanine and tryptophane. Increases in activity were observed after hybridization and further increases were observed after backcrossing in the case of endogenous respiration and tyrosine oxidation. It was suggested that the extra oxygen consumption of the resting cells was due to a tyrosinase activity.

Tryptophane and DOPA oxidation activity rose sharply in the first-generation hybrid and then fell to zero in the macromelanophores of the backcrossed hybrid. It was suggested that this might represent evidence that these two activities were carried on a chromosome different to that carrying the gene for tyrosine oxidation.

Phenylalanine activity in macromelanophores was unaffected by hybridization.

Hybrid and backcross melanomas both showed similar metabolic patterns, showing an increase in tyrosine and

phenylalanine activity with hybridization. Both DOPA and tryptophane activity were present in the tumor cells.

It was concluded that although tyrosine oxidation probably afforded a clue to the increased metabolism as hybridization progressed none of the activities tested played a causal role in tumor formation.

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THE BLOOD CHEMISTRY OF TERRESTRIAL AND AQUATIC SNAKES[1]

KENNETH E. HUTTON
Department of Biological Science, San Jose State College, San Jose, California

The physiology of reptiles has been studied to only a small extent, and, of the reptiles, the snakes have probably been studied the least. Although investigations have been made on the anatomy of the circulatory systems of these animals (see White, '55, '57), and erythrocytes have been counted and measured (see Wintrobe, '33; Bergman, '57), few studies have been otherwise concerned with their blood. Of the investigations which have dealt with the chemical components of snakes, those of Luck and Keeler ('29) and Carmichael and Petcher ('45) are probably the broadest.

In the present study the blood chemistry was investigated in snakes belonging to four species. Two of these species are common to aquatic habitats and two are found in terrestrial habitats. Thus it was hoped not only to obtain new data, but also to determine if the differences in habitats and habits of these snakes would be reflected in their physiology as indicated by their blood chemistry.

MATERIALS AND METHODS

The terrestrial snakes were Eastern yellow-bellied racers, *Coluber constrictor flaviventris,* and Eastern king snakes, *Lampropeltis getulus getulus.* The racers were collected in the vicinity of New Orleans; the king snakes were obtained from Ross Allen's Reptile Institute in Silver Springs, Florida, where they were collected locally. The aquatic snakes were

[1] This study was supported by a grant from the National Institutes of Health while at the Dept. of Zoology, Tulane University, New Orleans, Louisiana.

water moccasins, *Agkistrodon piscivorus,* and Florida water snakes, *Natrix sipedon pictiventris.* The moccasins were collected from swamps near New Orleans; the Florida water snakes were obtained from Ross Allen. The animals were kept in cages for two to three weeks before being bled. Lizards (*Anolis carolinensis*) and/or leopard frogs (*Rana pipiens*) were available to them for food except for 10 days prior to bleeding. Trays of water were constantly available within the cages.

The number of animals used, sex ratios, lengths and weights are indicated in the upper part of table 1. Except for *Agkistrodon,* blood was obtained by means of a cardiac puncture made with a number 22 needle while the snake was held on its dorsal surface. The blood was then injected into a beaker containing dry potassium oxalate as the anticoagulant. Due to the small size of the moccasins, cardiac punctures were not practical. Therefore the blood was obtained by cutting off the head and allowing blood from the body to drip into a beaker containing anticoagulant.

The blood constituents studied are listed in tables 1 and 2. Packed cell volumes were obtained by using the method of van Allen ('25), erythrocyte counts using the standard Neubauer counting chambers. Uric acid was determined according to the method of Folin ('34); the urea method was basically that of Gentzkow and Mason ('42). Except for sodium, all other biochemical determinations followed the techniques as put forth in the Manual of Standardized Procedures for Spectrophotometric Chemistry, edited by Fister ('50). Accordingly the lipoid phosphorus and inorganic phosphorus methods were basically that of Fiske and Subbarow, the chloride method was basically that of van Slyke and Hiller, the magnesium method basically that of Hirschfelder, and the determinations of glucose, total nitrogen, non-protein nitrogen, creatinine and amino acid nitrogen were based upon the methods and modifications of Folin and Wu. These colorimetric determinations were made on a Coleman Junior Spectrophotometer. Sodium was determined on a Beckman, model 4100, direct-reading flame photometer.

RESULTS

The findings of the quantitative determinations are listed in tables 1 and 2. In addition to the mean value and range of values for each component in each species, table 1 includes the standard error of the mean. The standard error is not included in table 2 because enough plasma was not always obtained for more than a few determinations in a species. No statistical comparisons were made on the data in table 2 due to the small number of samples in some cases. Statistically significant differences between species are noted in table 1 by means of superscripts. The lengths were not compared statistically since the aquatic snakes are characteristically stubbier than the terrestrial.

Although only the values obtained from the cardiac punctures of the racers are given in the tables, blood was also obtained by cutting off the heads — as with the moccasins. The values obtained by the two methods of procurement were compared. Except for glucose, the values were similar. The values for glucose obtained after cutting off the heads were approximately 30% higher than those obtained by means of the cardiac punctures. Probably this can be attributed to the excitement induced in the animals. If the findings of this comparison are transposed to the values for the moccasins, probably the values for glucose in the moccasins should be lowered somewhat.

DISCUSSION

The results (tables 1 and 2) indicate that the 4 species of snakes show significant differences only in a few components. Where differences are apparent, there are no patterns to indicate that these are differences between the terrestrial snakes and the aquatic snakes as such, but that these are simply individual species differences. This is interpreted as indicating that the blood chemistry does not show physiological differences correlated with the ecological differences. Apparently the 4 species of snakes are basically land ani-

TABLE 1

Characteristics of the whole blood of four species of snakes

		LAMPROPELTIS	COLUBER	NATRIX	AGKISTRODON [5]
Number and sex of animals		2♂, 3♀	2♂, 3♀	3♂, 2♀	3♂, 3♀
Length	Mean (cm)	122.5	131.3	93.8	54.8 [6]
	$S.E._m$	5.0	2.6	7.7	4.03
	Range	114.2–138.9	124.1–137.5	80.9–118.9	40.5–66.6
Weight	Mean (gm)	469	250	344	146 [1,3]
	$S.E._m$	66	15	83	33
	Range	260–650	210–275	145–650	40–310
Packed cell volume	Mean (%)	22.0	26.4	21.9	17.5 [2]
	$S.E._m$	2.9	2.9	2.5	1.1
	Range	14.0–31.6	22.0–31.0	16.0–28.0	14.0–20.5
Erythrocyte count	Mean ($\times 10^3$)	782.4	887.5	756.9	587.7 [2]
	$S.E._m$	81.6	65.7	59.2	41.8
	Range	538–1,027	730–1,075	570–885	468–697
Glucose	Mean (mg%)	60.4	77.0	48.9	49.2 [7]
	$S.E._m$	7.3	6.4	14.0	8.4
	Range	35.4–74.5	60.6–98.9	15.7–96.0	24.4–78.5
Creatinine	Mean (mg%)	1.40	0.60 [1,8]	1.46	0.90
	$S.E._m$	0.14	0.15	0.21	0.19
	Range	1.15–1.90	0.30–1.15	1.15–2.25	0.56–1.30

Lipoid-phosphorus	Mean (mg%)	16.4	16.6	10.8 [1,2,4]	15.9
	$S.E._m$	1.1	1.8	0.5	0.6
	Range	13.1–19.1	12.9–18.7	9.6–12.5	14.2–18.1
Total nitrogen	Mean (%)	1.21 [2]	1.95	1.46	2.84
	$S.E._m$	0.15	0.12	0.18	0.53
	Range	0.73–1.55	1.53–2.26	1.03–2.10	1.09–4.42
Non-protein nitrogen	Mean (mg%)	80.8	86.7	71.4	78.7
	$S.E._m$	10.2	3.0	3.2	7.9
	Range	48.0–109.5	80.0–97.0	61.4–78.5	60.4–87.7
Amino acid nitrogen	Mean (mg%)	40.6	44.2	36.0	36.9
	$S.E._m$	2.1	3.1	2.2	1.9
	Range	34.8–47.6	38.3–55.6	29.2–42.1	30.9–43.8
Uric acid	Mean (mg%)	5.51	5.69	6.02	5.47
	$S.E._m$	0.93	0.45	0.93	0.52
	Range	3.66–9.00	4.36–6.90	2.89–7.80	4.36–7.80
Urea	Mean (mg%)	2.03	3.61	1.58	4.89
	$S.E._m$	0.30	0.87	0.19	1.30
	Range	1.45–2.91	2.35–6.20	1.14–2.18	0.92–8.18

[1] A statistically significant difference from *Lampropeltis*.
[2] A statistically significant difference from *Coluber*.
[3] A statistically significant difference from *Natrix*.
[4] A statistically significant difference from *Agkistrodon*.
[5] Bled by decapitation instead of cardiac puncture.
[6] No statistical comparisons made regarding length.
[7] Note discussion in text.

TABLE 2

Inorganic ions in snake plasma

		LAMPROPELTIS	COLUBER	NATRIX	AGKISTRODON
Sodium	Mean (mEq/1)	1.24	1.41	1.16	1.41
	Range	1.12–1.35	1.31–1.50	1.08–1.32	1.25–1.57
	No. of determinations	5	4	3	6
Phosphorus	Mean (mEq/1)	7.10	11.61	5.06	5.12
	Range	3.89–16.20	7.86–17.50	4.69–5.81	5.19–8.37
	No. of determinations	5	4	3	6
Chloride	Mean (mEq/1)	79.5	71.4	64.2	88.5
	Range	58.9–102.0	64.0–78.0	59.1–71.5	80.9–102.0
	No. of determinations	5	4	3	5
Calcium	Mean (mEq/1)	4.9	6.1	4.6	6.7
	Range	3.9–6.0	3.9–8.6	4.0–5.9	4.5–10.0
	No. of determinations	5	4	3	5
Magnesium	Mean (mEq/1)	1.77	2.22	1.62	2.23
	Range	—	—	—	2.10–2.39
	No. of determinations	1	1	1	4

mals — as also indicated by methods of reproduction, respiration, and basking habits. Although *Natrix* and *Agkistrodon* are found in wet areas and catch prey in the water, their physiological adaptations are primarily those of land animals.

Comparisons may be made between findings here and other reported values on the blood chemistry of snakes. In general the results are similar to those obtained in the surveys by Luck and Keeler ('29) working with the rattlesnakes *Crotalus atrox* and *Crotalus viridis oregonus* and with the findings of Carmichael and Petcher ('45) working with *Crotalus horridus*. Although the packed cell volume and erythrocyte counts are somewhat lower than those of Carmichael and Petcher for *Crotalus horridus,* the erythrocyte counts are slightly higher than those found by Hecht et al. ('55) in the Bahaman piping boa, *Tropidophis pardalis.* Tipton ('33) found somewhat higher packed cell volumes and erythrocyte counts for *Natrix sipedon* and the garter snake *Thamnophis sirtalis.* The packed cell volumes reported by Rapoport and Guest ('41) for the moccasin and the king snake, as well as for the water snake *Natrix taxispilota,* fall within the ranges found in this study. Their values for inorganic phosphorus are also very similar to those found here.

Thoroughgoing work has been done with blood glucose in a few species of snakes. Rhaney ('48) found the blood glucose of *Lampropeltis getulus floridana* to range between 55 and 128 mg% with an average of 91 mg%. Prado ('46) found the blood sugar of *Philodryas sp.* to be 63 ± 19.4 mg% in 44 specimens. The same author, in 526 specimens of *Bothrops jararaca,* found that the glucose ranged between 30 and 130 mg% with an average of 67 mg% in males and 60 mg% in females.

Evaluations of the serum and plasma proteins in several species of snakes have been made elsewhere (Deutch and McShan, '49; Cohen, '54; Dessauer et al., '56). Protein levels in the present series of snakes may be calculated by the proper mathematical manipulations. However, the results are not strictly comparable since the work here was performed on

whole blood instead of serum or plasma. Non-protein nitrogen values reported here are decidedly higher than those reported by Luck and Keeler and Carmichael and Petcher in their work with rattlesnakes. This is due in part to the higher amino acid nitrogen found here. Urea values herein are intermediate between lower values reported by Luck and Keller and higher values reported by Carmichael and Petcher.[2] In the important comparison between urea and uric acid, the latter is found to be present in higher concentrations. Again, this reflects the physiological adaptations for terrestrial life found in all these snakes.

The values reported here are also of interest for taxonomic comparisons. *Agkistrodon* appears to have the greatest number of dissimilarities from the others. Perhaps this is a reflection of the fact that *Agkistrodon* is a member of the family Crotalidae whereas the other three species are members of the family Colubridae. If the values found here (particularly those for non-protein nitrogen, urea, and uric acid) are compared with reported values for other reptiles, it is found that they are more similar to lizards (Hernandez and Coulson, '51; Dessauer, '52) than to alligators (Hopping, '23; Coulson et al., '50) or turtles (Hutton and Goodnight, '57; also note footnote on this page). Thus relatively close relationship of the snakes with the lizards is indicated. This would tend to verify interpretations based upon immunological comparisons of reptiles (Cohen, '55) and morphological studies.

SUMMARY

Blood constituents were studied in two species of terrestrial snakes: the Eastern king snake, *Lampropeltis getulus*

[2] The method employed in the determination of urea appears to be of decisive importance in the values derived. Since publishing a previous paper on the blood chemistry of turtles (Hutton and Goodnight, '57), the author has found that the method of Gentzkow and Masen ('42) gives much more reliable results than the method then used and that the reported values for urea must be revised upward to a normal range of 9 to 35 mg% for the red eared turtle, *Pseudemys scripta elegans*, and of 16 to 43 mg% for the box turtle, *Terrapene carolina carolina*.

getulus, and the Eastern yellow-bellied racer, *Coluber constrictor flaviventris;* and in two aquatic species: the water moccasin, *Agkistrodon piscivorus*, and the Florida water snake, *Natrix sipedon pictiventris*. The constituents quantitatively evaluated included packed cell volume, erythrocyte count, glucose, creatinine, lipoid phosphorus, total nitrogen, non-protein nitrogen, amino acid nitrogen, uric acid, urea, sodium, chloride, magnesium, calcium, and inorganic phosphorus. No consistent differences were found between the aquatic and terrestrial forms, indicating that the "aquatic" snakes are largely terrestrial in physiological adaptation as well as development and habit. If the values, particularly those of the non-protein nitrogen components, are compared to those of other reptiles, they are found to be most similar to those of the lizards and indicate relationship with them.

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INTRACELLULAR WATER OF LARVAL TISSUES OF THE SOUTHERN ARMYWORM AS DETERMINED BY THE USE OF C^{14}-CARBOXYL-INULIN [1]

L. LEVENBOOK

Department of Biochemistry, Jefferson Medical College, Philadelphia

WITH THE TECHNICAL ASSISTANCE OF

JEANNE KUHN

ONE FIGURE

For any precise analysis of the distribution of a substance between the extracellular fluid and tissues of an organism, the relative concentrations must clearly be expressed in comparable, and preferably the same, units. Since most biological solutes such as free amino acids, sugars, inorganic salts, etc., are in aqueous solution, the concentration is best expressed in terms involving unit mass or volume of water.

Due to the lack of any information concerning the intracellular water content of insect tissues, previous studies on the distribution of free amino acids between insect blood and tissues (e.g., Florkin, '54; Duchateau and Florkin, '55) have generally involved comparison of the amount of solute in 100 ml of hemolymph on the one hand with 100 gm tissue on the other — a rough approximation at best. The absence of such information may be due in part to the fact that no experimental method for determining the intracellular water content of insect tissues has as yet been described. A procedure designed to achieve this end is accordingly presented in this paper.

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Pure intracellular fluid uncontaminated by interstitial fluid is, for most practical purposes, unobtainable. The intracellular water content of a tissue is therefore generally calculated as the difference between total tissue water and interstitial water. In insects, aside from a tubular dorsal heart, blood vessels are lacking and the blood bathes the tissues directly, and hence the interstitial and extracellular fluids form parts of the same fluid compartment. Since total tissue water may be readily determined by heat and/or vacuum drying, the problem resolves itself into a method for accurately measuring the tissue extracellular water content.

A number of procedures are available for the absolute measurement of extracellular water (cf. Elkinton and Danowski, '55); all of these are based on the assumed homogeneous distribution in solely the extracellular fluid of certain molecules such as thiocyanate, thiosulfate, sucrose, inulin, Cl^-, Na^{24} etc. The procedure to be described employs C^{14}-carboxyl-labeled-inulin, first suggested as a physiological tracer for inulin by Cotlove ('55).

EXPERIMENTAL

Materials. Fully grown 6th instar *Prodenia eridania* (Southern Armyworm) larvae were used for these experiments. The larvae were reared at 75°F and ca. 90% relative humidity on a diet first of bean seedlings and subsequently of potato slices. Mature, average sized larvae were selected, abnormally large or small specimens being rejected.

C^{14}-carboxyl-labeled inulin, with a specific activity of 0.54 μc per mg was obtained from Dr. H. S. Isbell, National Bureau of Standards, Washington, D. C.

Procedure. The *Prodenia* larvae were anaesthetized with carbon dioxide (Williams, '46) and injected with a known volume, generally 5 μl, of a 5% (w/v) aqueous solution of C^{14}-inulin (about 150,000 cpm). Injections were performed under × 10 magnification, employing fine glass needles cemented to No. 28 hypodermic needles, and an "Agla" micrometer syringe assembly (Burroughs, Wellcome Ltd., England) supported by a rack and pinion device for controlling precise

lateral motion. The tip of the glass needle was inserted into the apex of a proleg around the base of which was a loose ligature of fine nylon thread. As the needle was withdrawn following injection of the desired volume, a second operator tightened the ligature, thereby preventing loss of any fluid from the larva.

At varying intervals of time after injection with C^{14}-inulin, the larvae were again anesthetized, an incision was made in another proleg, and exactly 5 μl samples of hemolymph were aspirated into a Lang-Levy constriction pipette. These samples were plated out directly onto stainless steel planchets, dried under an infra-red lamp, and counted for C^{14}. A suitable volume of hemolymph was also taken for determination of its water content by drying to constant weight at 110°C.

The blood samples having been obtained, the larvae were next dissected in an apolar, non-toxic liquid, trichlorotrifluoroethane (Freon 113).[2] This solvent, being almost completely immiscible with water (the solubility of water in Freon 113 at room temperature is about 0.01% by weight), does not disturb the water relationships between the extra- and intracellular fluids. Freon 113 was found preferable in every respect to carbon tetrachloride, which was employed in preliminary experiments. The entire gut and most of the fat-body were dissected out, the former was freed of its internal contents, and the tissues were rinsed in fresh Freon 113 prior to being superficially dried on Whatman No. 50 filter paper to remove adhering solvent or hemolymph. The individual tissues thus obtained were divided into two approximately equal portions, and each was weighed rapidly on a torsion balance. The total water content of the one portion was determined by drying to constant weight at 110°C and the other was employed for measurement of the extracellular C^{14}-inulin content, as follows.

The tissue (5–18 mg wet weight) was transferred with a platinum wire loop to a small (0.5 ml volume) conical shaped tube containing 100 μl of 10% (w/v) KOH. After digestion

[2] E. I. duPont de Nemours, Wilmington, Delaware.

for 20–30 minutes on a boiling water bath and cooling to room temperature, the tube was placed on ice. The KOH was neutralized with 72% perchloric acid, the precipitated $KClO_4$ centrifuged down in the cold, and the supernatant fluid transferred to a tube calibrated at 200 µl. The precipitate was washed twice on the centrifuge with cold water, and the combined supernatant and washings were made up to 200 µl. In this manner some 96–99% of the tissue total counts was recovered in the volumetric tube.

For measurement of radioactivity, duplicate 10 or 20 µl aliquots, together with a drop of the wetting agent Triton-X100[3] diluted 1:500, were placed on a stainless steel planchet, and the solution was spread evenly over the surface with a platinum wire. After drying the planchet under an infra-red lamp, the counts were recorded in the conventional manner, employing a windowless gas-flow Geiger tube. Under the conditions employed, the counter had an efficiency of close to 40%, and the statistical error of counting did not exceed 3%.

From the values determined by the above procedure, the following calculations could be made:

$$\text{Total extracellular fluid volume of injected larva (in } \mu\text{l)} = \frac{(\text{cpm* per } \mu\text{l C}^{14}\text{-inulin inj.})\ (\mu\text{l C}^{14}\text{-inulin sol. inj.}) - (\mu\text{l C}^{14}\text{-inulin sol. inj.})}{(\text{cpm per } \mu\text{l hemolymph after t hours equilibration})} \qquad (1)$$

* cpm = counts per minute.

$$\text{Total tissue water (per cent of tissue wet weight)} = \frac{(\text{tissue wet weight, mg}) - (\text{tissue dry weight, mg})}{(\text{tissue wet weight, mg})} \times 100 \qquad (2)$$

$$\text{Tissue extracellular water (per cent of tissue wet weight)} = \frac{(\text{cpm in aliquot of HClO}_4\text{ extract})}{(\text{cpm per mg hemolymph H}_2\text{O})\ (\mu\text{l HClO}_4\text{ extract aliquot})} \times \frac{(\text{total volume of HClO}_4\text{ extract})}{(\text{wet weight of tissue, mg})} \times 100 \qquad (3)$$

$$\text{Tissue intracellular water (per cent of tissue wet weight)} = \text{Equation (2)} - \text{Equation (3)} \qquad (4)$$

[3] Rohm and Haas Co., Philadelphia, Pa.

RESULTS

In order to judge the probable validity of the proposed method for measuring tissue extracellular water, a number of likely criteria, enumerated below, were first examined.

1. *No radioactivity is lost by hot KOH digestion.* The satisfactory recovery of C^{14} after boiling C^{14}-carboxyl-inulin with 10% KOH was demonstrated as follows. Twenty µl of a C^{14}-inulin solution having 160 cpm per µl were added to 100 µl of 10% KOH, heated on a boiling water bath for 30 min., and carried through the procedure described above under "Experimental." In duplicate determinations, 96% and 99% of the initial 3200 cpm were finally recovered.

2. *No inulase, or other enzyme attacking inulin, is present in Prodenia larva hemolymph.* One-hundred µl of hemolymph were diluted with an equal volume of M/30 pH 6.8 phosphate buffer and a drop of toluene, and incubated for 4 hours at 37°C with either 1.0 mg inulin or 1.0 mg fructose as substrates. Boiled hemolymph provided appropriate controls. After incubation, the solutions were deproteinized, concentrated, and subjected to paper chromatography using butanol-acetic acid-water (Partridge, '48). No increase in fructose could be detected following incubation of the hemolymph with inulin. Furthermore, as assayed by the method Roe, Epstein and Goldstein ('49), inulin could be recovered quantitatively following its incubation with hemolymph.

3. *C^{14}-carboxyl-inulin is not bound by Prodenia hemolymph proteins.* A *Prodenia* larva was injected with 150,000 cpm of C^{14}-inulin. The following day a 50 µl sample of hemolymph (corresponding to about 40,000 cpm) was deproteinized by the addition of 0.5 ml of saturated ammonium sulfate. The precipitated protein was washed 6 times on the centrifuge with 0.5 ml portions of saturated $(NH_4)_2SO_4$, and the washing continued with successive 0.5 ml volumes of 70%, 95% and 100% ethanol, and finally with ether. The resulting protein pellet was then air-dried. Only a negligible number of

counts (20 or less) was found in either the last $(NH_4)_2SO_4$ or 70% ethanol washing, or in the protein itself.

A second larva was also injected as above, and a 50 µl hemolymph sample subjected to paper electrophoresis. Two protein bands were separated in veronal buffer pH 8.5 after running at 200 v for 12 hours. Neither of these bands was associated with any radioactivity. These two experiments appear to indicate, therefore, that C^{14}-inulin is not bound by *Prodenia* hemolymph proteins.

4. C^{14}-inulin is neither excreted nor metabolized by Prodenia larvae. Two larvae were starved overnight and then each was injected with ca. 150,000 cpm of C^{14}-inulin. Following the injection, both larvae were placed in a small, airtight bottle lined with damp filter paper. The bottle was equipped with a center well containing 1.0 ml of 4N NaOH, which was covered with a wire gauze. Carbon dioxide-free air was admitted into the bottle through a capillary tube attached to a soda-lime tube. After 12 hours at 75°F the respiratory carbon dioxide trapped in the alkali was examined for radioactivity by precipitation of the CO_2 as $BaCO_3$. The excrement and filter paper were extracted with boiling water, the extract was centrifuged and concentrated, and aliquots plated out directly for radioactive counting. The combined counts from both of these sources were less than 5% of the counts injected, from which it may be concluded that the C^{14}-inulin was not excreted, and is metabolically inert.

In contradistinction to the above findings, C^{14}-inulin injected directly into the mid-gut was both oxidized and excreted. This would appear to offer at least prima facie evidence that inulin, when injected directly into the hemolymph, does not penetrate the larval *Prodenia* cells.

As shown in figure 1, which summarizes all the data, the values for the intracellular water of the fat-body and gut are substantially the same from 2.75 hours onwards following the injection of C^{14}-inulin.

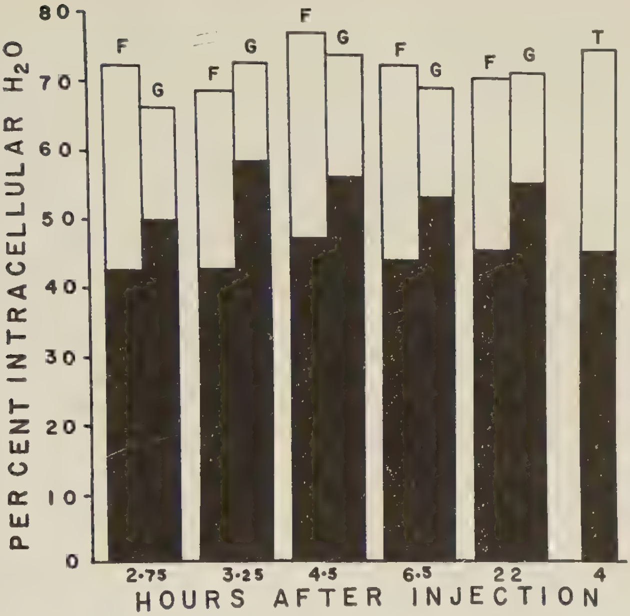

Fig. 1 Intracellular water content of larval *Prodenia* tissues. Each of the columns above the indicated hours after injection of C^{14}-inulin represents the mean value for two larvae. The total height of each column denotes intracellular water as per cent of the total tissue water, the solid column as per cent of tissue wet weight. F = fat-body; G = gut; T = combined internal tissues excluding cuticle.

TABLE 1

Intracellular water content of larval Prodenia tissues

TISSUE	PER CENT INTRACELLULAR WATER	
	of tissue wet weight	of tissue total water
Fat-body	44.6 ± 1.8 (S.D.) [1]	72.3 ± 3.1 (S.D.)
Gut	54.8 ± 3.3	70.9 ± 2.7
Combined internal tissues	45.5	71.5

[1] Standard deviation of the mean for the five pairs of larvae.

This would indicate a surprisingly rapid equilibration of the polysaccharide within the larval extracellular space, especially since an anesthetized larva takes at least 30 minutes to recover from the effects of the carbon dioxide. In calculating the intracellular water, therefore, it appeared justifiable

to combine all the data of figure 1 to obtain the average values, which are presented in table 1.

It will be noted that whereas intracellular water makes up approximately the same proportion of the total tissue water of all the tissues examined, when expressed as a percentage of the tissue wet weight the value for fat-body is considerably lower than that for gut. This is undoubtedly due to the high fat and glycogen content of the fat-body cells (Babers, '41). In view of the large bulk of the fat-body relative to its contribution to the combined internal tissues, the similarity of

TABLE 2

Extracellular fluid volume of 6th instar Prodenia larva

TIME AFTER INJECTION OF C^{14} INSULIN	EXTRACELLULAR FLUID VOLUME (Mean of two larvae for each time)
Hours	*ml*
2.75	0.178
3.25	0.203
4.00 [1]	0.173
4.50	0.207
6.50	0.183
22.00	0.221
Mean	0.188 ± .016

[1] These specimens were not included in the determination of the tissue intracellular water content.

their respective contents of intracellular water is not unexpected.

As indicated by equation (1), a knowledge of the volume and total counts of C^{14}-carboxyl-inulin solution injected provides an easy procedure for measuring the total extracellular fluid volume. Employing the identical larvae used for determination of tissue intracellular water, the values for total extracellular fluid volume of these larvae are presented in table 2. In the absence of evidence to the contrary, the insect extracellular fluid and hemolymph may be considered to be parts of the same fluid compartment occupying, in the average sized

Prodenia larva, a volume of about 0.19 ml. Babers ('38) states that the volume of blood obtainable from a mature *Prodenia* larva varies from 0.07 to 0.2 ml, with an average of 0.12 ml.

DISCUSSION

Inulin has frequently been employed for measurement of extracellular space in vertebrates (see Keith, '53 and Siri, '56 for reviews and references). The space so estimated is generally smaller than that determined by the use of chloride, Na^{24} etc., unless a prolonged time is allowed for the very slow diffusion of inulin into collagenous connective tissues (Nichols et al., '53; Cotlove, '54). There is little, if any, comparable connective tissue in insects, and no such slow diffusion into the soft insect tissues has been observed. The diffusion of C^{14}-carboxyl-inulin into *Prodenia* tissues is in fact quite rapid, being complete in less than three hours.

The accuracy of the present values for the intracellular water content of larval *Prodenia* tissues depends largely upon the validity of the proposed procedure for measuring extracellular water. The procedure itself rests upon the two assumptions that inulin, and hence presumably C^{14}-carboxyl-inulin, does not penetrate cells and is homogeneously distributed throughout the extracellular space. Neither of these assumptions has been, nor indeed can be, tested directly in *Prodenia;* but they are compatible with all the "circumstantial criteria" examined in judging whether these assumptions do in fact apply to the *Prodenia* larva.

Attention may be directed to a possible error in the tissue wet weight, due to the superficial drying of the tissue to remove contaminating Freon 113 or hemolymph. The exact wet weight of the fat-body is difficult to determine, due to the extremely loose nature of the tissue. In the case of the gut, however, a particular difficulty was the fact that treatment with Freon 113 (but not with aqueous media) resulted in the formation of an easily removable yellowish, gelatinous material on the inner surface. This material, the dry weight of which did not exceed a few per cent of that of the entire gut,

to a greater or lesser extent remained attached to the filter paper during the drying process, and was not included in the gut weight.

Blood dilution methods for measuring the blood volume of insects have been described by Beard ('49) and Yeager and Munson ('50). C^{14}-carboxyl-inulin offers an alternative, and possibly less equivocal, procedure. The average weight of a mature *Prodenia* larva is about 0.7 gm; the total extracellular fluid volume of 0.19 ml thus amounts to ca. 27% of the body weight, a value close to that reported for the *Bombyx mori* larva by Richardson, Burdette and Eagleson ('31).

SUMMARY

1. C^{14}-carboxyl-inulin, injected into the 6th instar *Prodenia eridania* (Southern Armyworm) larva, can be recovered quantitatively from the blood, is not bound by the hemolymph proteins, and is neither metabolized nor excreted.

2. A method employing C^{14}-carboxyl-inulin for the determination of tissue extracellular water, and hence the intracellular water content of insect tissues, is described. The method serves also to measure the total extracellular fluid volume of insects.

3. Values are presented for the intracellular water content of the fat-body, gut and combined internal tissues of the mature *Prodenia eridania* larva, together with measurements of the total extracellular fluid volume.

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THE OXYGEN-RESPIRATORY PIGMENT EQUILIBRIUM OF THE HEMOCYANIN AND MYOGLOBIN OF THE AMPHINEURAN MOLLUSC *CRYPTOCHITON STELLERI*

CLYDE MANWELL[1]

Department of Biological Sciences, Stanford University, and Marine Field Laboratories of the University of Washington, Friday Harbor

TWO FIGURES

There are two systems in which respiratory pigments appear to be specialized for the purpose of increasing the efficiency of the diffusive transfer of oxygen from one biological phase to another: (1) the transfer of oxygen from the hemoglobin of the blood to the hemoglobin of the muscles — myoglobin (Millikan, '39); and, (2) the transfer of oxygen across the placenta from maternal to fetal hemoglobin (Barcroft, '47; Lamport, '54). Studies have indicated that in both of these cases the oxygen dissociation curve of the "acceptor respiratory pigment" (myoglobin or fetal hemoglobin) lies to the left of that for the corresponding "donor respiratory pigment," i.e., the oxygen affinity of the "acceptor pigment" is greater than that of the "donor pigment." The greater this difference in the oxygen dissociation curves, the larger the amount of oxygen per unit time that will diffuse across the barrier separating the two respiratory pigments. This increased efficiency results from the ability of the "acceptor respiratory pigment" to become appreciably saturated with oxygen at low tensions of that gas. Consequently, oxygen tension on the acceptor side of the barrier remains low, thereby increasing the magnitude of the oxygen gradient, and, thus, the net diffusive transfer of dissolved oxygen.

[1] Present address: Department of Experimental Biology, University of Utah, Salt Lake City 12, Utah.

Only the oxygen transfer systems in the vertebrates have been studied with respect to the location of the oxygen dissociation curves. In the invertebrates there are several examples of juxtapositions of different respiratory pigments that are suggestive of potential oxygen transfer systems. (Some of these are mentioned in H. M. Fox, '49, '55.) However, the relative position of the oxygen dissociation curves of these juxtaposed respiratory pigments has not been determined except in the case of the polychaete *Nephthys hombergii* (Jones, '54); that the coelomic and vascular hemoglobins of this polychaete actually participate in an oxygen transfer system is, according to Jones, uncertain, for not enough is known of the anatomical relationships between the blood vessels and the coelom in this particular species. One of the most wide-spread juxtapositions of different respiratory pigments is seen in amphineuran and gastropod molluscs; in many of these forms the radular muscles possess myoglobin, whereas the blood contains hemocyanin (Lankaster, 1873; Ball and Meyerhof, '40; Ball and Cooper, '47; D. L. Fox, '53). In order to ascertain whether or not this combination of hemocyanin and myoglobin functions as an oxygen transfer system it is necessary to know the oxygen dissociation curves of both respiratory pigments under physiological conditions, as well as the anatomical relationship between the two pigments. For this purpose studies have been made on the oxygen equilibrium of the respiratory pigments of the chiton *Cryptochiton stelleri* Middendorff, the presence of hemocyanin (Stewart, Dandliker, and Martin, '52) and radular myoglobin (Giese, '52) having been demonstrated by spectrophotometric analysis. Oxygen dissociation curves have not been published for any amphineuran hemocyanin or molluscan myoglobin.

MATERIALS AND METHODS

Preparation of the respiratory pigments

Clean specimens of *Cryptochiton* were opened ventrally in the region of the branchial sinuses; the hemocoelic fluid ob-

tained thereby was filtered first through glass-wool and then through Whatnam # 1. As with other molluscs the blood does not clot. Without further treatment this hemocyanin solution was used within an hour for determination of its oxygen dissociation curve.

Excised muscles of the odontophore complex, bright red from the high concentration (1–2%) myoglobin, were homogenized in an equal volume of distilled water, or, in a few cases, potassium phosphate buffer ($pH = 7.5$; $\Gamma/2 = 0.2$). The homogenate was allowed to stand overnight in the refrigerator, after which it was centrifuged and filtered. The resultant myoglobin solution was diluted with an equal volume of potassium phosphate buffer of appropriate pH and $\Gamma/2 = 0.40$. Approximately 1 cm^3 of a 0.5–1.5% myoglobin solution was obtained from each specimen.

As the purpose of these experiments was to obtain the oxygen dissociation curve under conditions as physiological as practical, further purification of the respiratory pigments was not attempted. The importance of this will become more apparent when the shape of the oxygen dissociation curve of the myoglobin is considered. All steps in the preparation of the hemocyanin and the myoglobin were done at 0°C. Both pigments are more unstable than most respiratory pigments; a few hours at 20–25°C results in a loss of much of their oxygen-combining capacity, the myoglobin being converted to metmyoglobin, the hemocyanin to some unknown derivative.

Determination of the oxygen dissociation curves

In order to work rapidly with relatively small quantities of respiratory pigment a new modification of standard spectrophotometric techniques for measuring the oxygen-respiratory pigment equilibrium has been devised.

A small test-tube (3 cm^3) containing 1 cm^3 of respiratory pigment solution was attached by means of a rubber stopper to the end of separatory funnel (75 cm^3) opposite from the stop-cock. The oxygen tension was varied by injection of

known quantities of air or pure O_2, as in the technique of Riggs ('51). The "per cent saturation" (per cent oxyhemocyanin or per cent oxymyoglobin) was determined spectrophotometrically in the usual manner (Lemberg and Legge, '49), a special cuvette holder enabling one to use a Beckman Model DU Spectrophotometer; "λ" = 600–650 mμ for hemoglobin, 600 mμ for hemocyanin. Care was taken to assure that at any particular oxygen tension equilibrium between oxygenated and deoxygenated forms of the respiratory pigment had been reached.

In general the pH of the respiratory pigment solution was varied through the addition of potassium phosphate buffers ($\Gamma/2 = 0.40$). However, when dilution of the hemocoelic fluid was to be avoided, the preparation was buffered by working under constant carbon dioxide tension, the desired volume of CO_2 being injected before the initial equilibration with N_2. The partial pressure of CO_2 was calculated by a formula analogous to that used for the partial pressure of O_2 (Riggs, '51); the appreciable volume of CO_2 that can be dissolved was compensated for by repeating the equilibration twice. The pH was evaluated by glass electrode; for *Cryptochiton* hemocoelic fluid equilibrated with a definite partial pressure of CO_2 it was necessary to make the pH determination rapidly and without agitation of the fluid. With such precautions there was no significant loss of CO_2 from — and, thus, rise in the pH of — hemocoelic fluid.

All oxygen-respiratory pigment equilibria were measured at 10°C, an approximate average of the temperatures to which *Cryptochiton* is normally exposed.

RESULTS

A comparison of the oxygen dissociation curves of *Cryptochiton* hemocyanin and myoglobin is shown in figure 1. It is clear that the curve for myoglobin lies well to the left of that for the hemocyanin.

It is also evident from this figure that the oxygen-hemocyanin equilibrium is relatively insensitive to changes in the

pH, at least in the range of physiologically potential pH's (6.5–7.5) at which experiments were done. Attempts to extend this pH range were not successful due to the instability of the hemocyanin. A slight movement of the oxygen dissociation curve to the left appears to take place at pH's greater than 7.7, possibly due to denaturation of the protein.

Experiments on various *Cryptochiton* myoglobin preparations indicate that the oxygen dissociation curve is hyperbolic

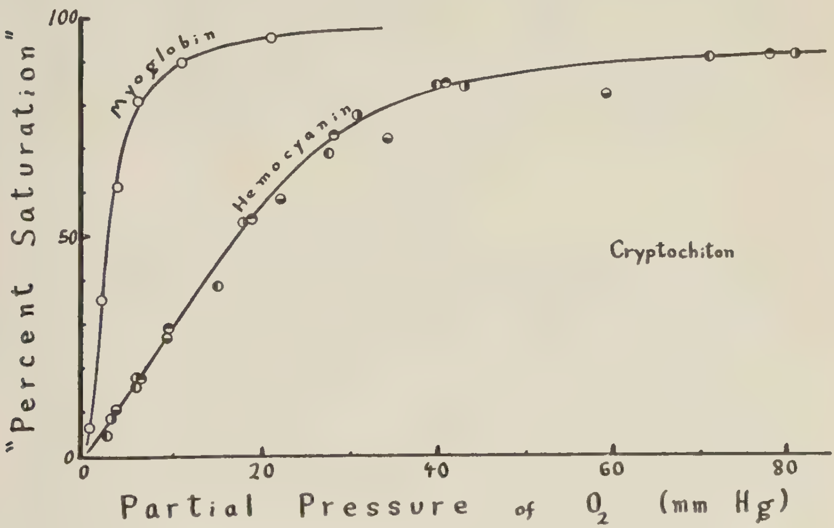

Fig. 1 Oxygen dissociation curves of the myoglobin and hemocyanin of the amphineuran mollusc *Cryptochiton stelleri.* General comments: temperature = 10°C; both pigments from the same specimen.
Explanation of symbols:

Radular myoglobin

○ pH = 7.3; potassium phosphate buffer; final ionic strength = 0.2.

Hemocyanin

Undiluted hemocoelic fluid under constant partial pressure of carbon dioxide (pCO_2).

◐ pH = 6.84; pCO_2 = 25 mm Hg.

◒ pH = 6.42; pCO_2 = 75 mm Hg.

3 parts hemocoelic fluid: 1 part potassium phosphate buffer. No carbon dioxide.

◑ pH = 7.30 ◓ pH = 7.10

when the extraction is made in distilled water; however, a sigmoid oxygen dissociation curve is observed when the pH is kept from falling much below neutrality during the extraction process by addition of a small amount of potassium phosphate buffer. Data on the oxygen equilibrium of *Cryptochiton* myoglobin is best expressed in terms of Hill's equation, which although originally derived under an incorrect assumption (Lemberg and Legge, '49), represents an algebraic approximation to the solution of a problem concerning the percentage of occupied sites on a multivalent protein capable of binding ligands reversibly in such a manner that combination at one site influences the affinity of other sites (see, for example, Klotz, '54). Substituting "p_{50}^{-n}" for the usual equilibrium constant, "K," Hill's equation becomes

$$y = 100 \frac{(p/p_{50})^n}{1 + (p/p_{50})^n}$$

"p" and "y" are the partial pressure of O_2 (mm Hg) and the "per cent saturation" respectively. "p_{50}" is the oxygen tension at which exactly half of the respiratory pigment is in the oxygenated form. "n" is a measure of the interaction between the oxygen-affine centers. In order to evaluate the constants "p_{50}" and "n" the usual practice is to plot "log y/(100 — y)" as a function of "log p." The result is a straight line (at least for 10 < "y" < 90) whose slope is "n;" "p_{50}" is that value of "p" for which "log y/(100 — y)" is zero.

The result of the application of this transformation to certain *Cryptochiton* myoglobin data can be seen in figure 2. From figure 2 it is apparent that when the original phosphate buffered extraction was allowed to stand over-night in the refrigerator under a N_2 atmosphere much but by no means all of the heme-heme interactions had disappeared. Hence, under certain conditions extremely labile heme-heme interactions are present in *Cryptochiton* myoglobin. It is entirely possible — but far from proved — that the oxygen dissociation curve of the pigment *in situ* is sigmoid. However, as the oxygen dissociation curve of the myoglobin is only changed

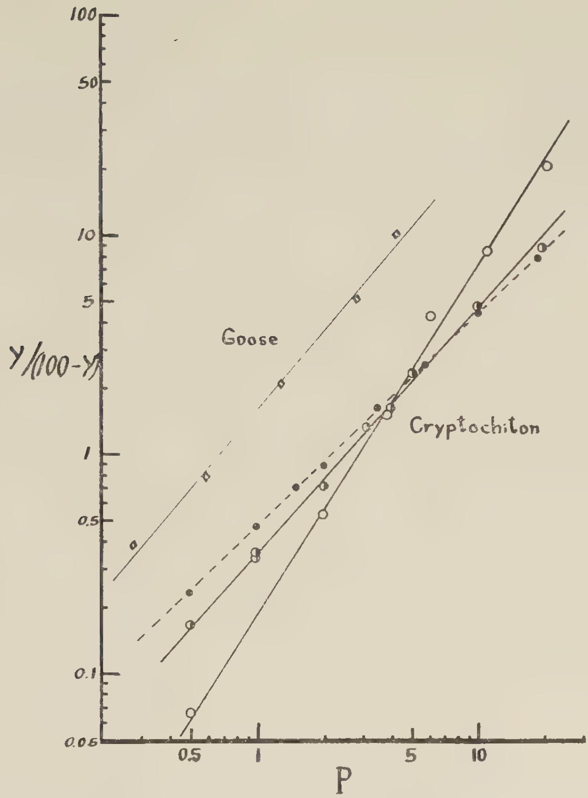

Fig. 2 The oxygen-myoglobin equilibrium. General comments: temperature $= 10°C$; myoglobin concentration $= 1\%$; potassium phosphate buffer; ionic strength $= 0.2$.

Explanation of symbols:

Solid lines — myoglobin extracted at $pH > 7$

○ original preparation; $pH = 7.30$

◑ same preparation; 24 hours later; $pH = 7.39$

Dashed lines — typical example extracted at $pH = 6.0–6.5$.

● $pH = 6.98$

Data on goose myoglobin from Hill ('36).

in shape but not shifted towards the oxygen dissociation curve of the hemocyanin, the possibility of heme-heme interaction in *Cryptochiton* myoglobin in no way invalidates the previous conclusion that the hemocyanin and the myoglobin of the animal are able with respect to the relative locations

of their oxygen dissociation curves to participate in an oxygen transfer system.

Data presented in table 1 indicates the magnitude of "n" observed in different preparations and shows that what little Bohr effect this muscle hemoglobin possesses is "reverse" at physiological pH's.

TABLE 1

The oxygen equilibrium of Cryptochiton myoglobin

TEMPERATURE (° C)	pH	"n"	"p_{50}"	NUMBER OF DETERMINATIONS
Cryptochiton No. 1 — preparation extracted at neutrality				
10.0	7.30	1.63	2.80	6
Cryptochiton No. 2 — same preparation, 24 hours later				
9.5	7.39	1.13	2.45	9
Other preparations — more acidic extractions				
10.1	7.90	0.99	3.01	7
10.0	7.44	0.98	2.57	7
10.0	7.00	1.02	2.32	7
10.1	6.98	0.99	2.11	8
10.0	6.52	1.00	2.61	6
10.0	5.92	0.95	2.14	6

DISCUSSION

The oxygen transfer system

Lankaster (1873) noted that myoglobin tends to occur in those muscles involved in prolonged intermittent activity. Such muscles would not obtain oxygen as easily when contracted as when relaxed, not only because of shape changes but, in the case of vertebrates at least (because of decrease in the circulation through a contracted muscle. Therefore, in such cases the high oxygen affinity of the myoglobin enables the acquisition by diffusion of a sufficient quantity of oxygen during the relaxed phase, as well as the gradual release of this stored oxygen during contraction.

The results of the studies on the respiratory pigments of *Cryptochiton* indicate that these pigments are suitable with respect to their relative oxygen affinities to participate in oxygen transfer. Concerning the anatomical relationship between the hemocoelic fluid and the myoglobin-containing muscles, the following short description is based on Heath ('05) and original observations. Many of the radular muscles originate on or very near the ventral surfaces of the first two anterior valves (plaques) and insert on various portions of the odontophore complex. These radular muscles consist primarily of two main groups, each comprised of many thin (1 mm or less) muscle bands of similar origins and insertions. It is assumed that these two main groups operate antagonistically in the movement of the radula back and forth as *Cryptochiton* browses on various algae. Blood vessels carrying oxygenated blood from the heart open into many parts of the hemocoele in which these radular muscles are located. The subdivision of the muscles into numerous strands allows the blood to circulate past a much greater surface than would be present if there were only a few compact muscle bundles. Hence, this arrangement indicates that there is ample opportunity for the two respiratory pigments to be sufficiently close to facilitate the transfer of oxygen into the muscles.

The author has found radular hemoglobin in a large number of amphineurans and gastropods, even such small limnic forms as *Planorbis, Limnaea,* and *Physa.* However, the terrestrial pulmonates *Helix aspersa* and *Agriolimax columbianus* and the carnivorous tectibranch *Navanax inermis* lack myoglobin. In these various molluscs hemoglobin is present in very small concentration, if at all, in the heart and other tissues; however, *Tivella stultorum,* the pismo clam, has hemoglobin in several of its tissues (D. L. Fox, '53).

Comparison of some properties of a molluscan and mammalian myoglobins

While *Cryptochiton* and mammalian myoglobins (information on the latter based on Theorell, '34b; Hill, '36; Millikan,

'39) are similar in their high oxygen affinities and their location in rhythmically contractile tissue, they appear to differ in two respects: (1) Horse and ox myoglobins have a small normal Bohr effect; *Cryptochiton* myoglobin has an extremely small Bohr effect that is "reverse" in nature. (2) Whereas the oxygen dissociation curves of horse, dog, ox, and sheep myoglobins are hyperbolic (i.e., "n" = 1.00), the same is not always true in the case of *Cryptochiton* myoglobin.

This last point, however, warrants further comment. The presence of heme-heme interactions in *Cryptochiton* myoglobin indicates that least some of the molecules consist of two or more oxygen-affine centers under certain conditions. Hence, as the basic "unit" of various hemoglobins has a molecular weight of approximately 17,000 (Haurowitz and Hardin, '54), these particular *Cryptochiton* myoglobin molecules must have a molecular weight of at least 34,000. While mammalian myoglobin is believed to have but a single oxygen-affine center and a molecular weight of 17,000, there is some evidence to the contrary. Theorell ('34a) observed on certain but not all occasions sedimentation constants for horse myoglobin indicating a molecular weight of 34,000; however, his ('34b) oxygen dissociation curves are hyperbolic. However studies by Kiese and Kaeske ('42) on the equilibrium of horse metmyoglobin with various anions (azide, cyanide, etc.) indicate that heme-heme interactions can also occur in a mammalian myoglobin. Finally, transforming data of Hill ('36) for the oxygen equilibrium of goose myoglobin, shown in figure 2 of this paper, indicates a value of "n" = 1.22, significantly higher than that found for various mammalian myoglobins. Unfortunately, the oxygen dissociation curve of no myoglobin has been done with the respiratory pigment *in situ* (Lemberg and Legge, '49). Although the possibility of contamination of vertebrate myoglobin with hemoglobin from the blood exists, this cannot occur in the case of *Cryptochiton*.

Cryptochiton hemocyanin appears to lack a Bohr effect in the physiological pH range; this is in contrast to all other

sufficiently investigated hemocyanins. The absence of a Bohr effect has been found to be characteristic of the hemerythrin of *Phascolosoma (Physcosoma) agassizii*, and of the hemoglobins of the polychaete *Eupolymnia crescentis* and the holothuroidean *Cucumaria miniata* (Manwell, unpublished observations); the possible significance of this peculiarity will be discussed when these additional new data are presented.

SUMMARY

Oxygen dissociation curves for the hemocyanin and myoglobin of the amphineuran *Cryptochiton stelleri* have been obtained. It appears as if these pigments function in an oxygen transfer system analogous to that involving blood and muscle hemoglobins, or maternal and fetal hemoglobins in various vertebrates. Under certain conditions heme-heme interactions are observed in *Cryptochiton* myoglobin.

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